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Dated: May 14, 2007
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Docket No.: AO-UTSC:791US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Mien-Chie Hung et al.

Application No.: 10/816,698

Confirmation No.: 1150

Filed: April 2, 2004

Art Unit: 1642

For: ANTITUMOR EFFECT OF MUTANT BIK

Examiner: Goddard, Laura B.

APPEAL BRIEF

Commissioner for Patents
Washington, D.C. 20231

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MS Appeal Brief
Commissioner of Patents
Washington, D.C. 20231

Sir:

Appellants hereby submit an Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated October 31, 2006 (the "Action"). The Notice of Appeal was filed on February 13, 2007. This submission is accompanied with a Petition for Extension of Time of One Month and the requisite fee.

The fee for filing this Appeal Brief is $250.00. Appellants assert that an additional fee is not required, but if this is in error, please charge the Deposit Account 06-2375 under the reference number AO-UTSC:791US, from which the undersigned is allowed to withdraw.

I. REAL PARTIES IN INTEREST

The real parties in interest are the assignee, Board of Regents, The University of Texas System, and the licensee, Alchemgen Therapeutics Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-11, 13, 17, and 43-75 are canceled. Claims 27-40 are withdrawn. Claims 12, 14-16, 18-26, and 41-42 are under examination and are the subject of appeal.

IV. STATUS OF AMENDMENTS

There are no amendments.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention generally concerns methods of inducing anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in a subject, comprising administering to the subject a mutant Bik polypeptide having an altered amino acid sequence, relative to SEQ ID NO:3, that comprises a substitution at least at a mutation at Thr^{33} or Ser^{35}, wherein the mutant Bik polypeptide induces anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in the subject, as represented in claim 12 and that finds support in the specification at least in paragraph **[0007]**, for example. In certain cases, the substitution is a Thr^{33} to Asp^{33} substitution, as represented by claim 14 and that finds support in the specification at least in the original claims and at paragraph **[0027]**, for example. In some embodiments, the substitution is a Ser^{35} to Asp^{35} substitution as represented by claim 15 and that finds support in the specification at least in the original claims and at paragraph **[0027]**, for example.

In some embodiments of the invention, the polypeptide further comprises a protein transduction domain, as represented by claim 16 and that finds support in the specification at least in the original claims and at paragraph **[0028]**, for example. In some aspects of the invention, the subject is a human or has a proliferative disorder, such as cancer, as represented respectively by claims 18, 19, and 20 and that finds support in the specification at least in the original claims and at paragraphs **[0006]** and **[0031]**, for example.

In specific cases of the invention, the cancer is breast cancer, prostate cancer, ovarian cancer, sarcoma, lung cancer, brain cancer, pancreatic cancer, liver cancer, bladder cancer, gastrointestinal cancer, leukemia, lymphoma, or myeloma, as represented by claim 21 and that finds support in the specification at least in the original claims and at paragraph **[0011]**, for example. In some aspects of the invention, the cancer is estrogen receptor positive, is EGF receptor overexpressing, is Her2/neu-overexpressing, is not Her-2/neu-overexpressing, is Akt overexpressing, is angrogen independent, or is androgen dependent, whereas in other aspects, the cancer is a solid tumor, such as, for example, sarcoma, lung, brain, pancreatic, liver, bladder, gastrointestinal cancers, or hematologic malignancies, such as leukemia, lymphoma, and myeloma, respectively, which are represented by claims 22 and 23 and that find support in the specification at least in the original claims and at paragraph **[0011]**, for example.

In some embodiments of the invention, the proliferative cell disorder is restenosis, as represented by claim 24 and that finds support in the specification at least in the original claims and at paragraph **[0022]**, for example. In certain aspects of the invention, the polypeptide is comprised in pharmacologically acceptable excipient, as represented by claim 25 and that finds support in the specification at least in the original claims and at paragraph **[0027]**, for example, or the polypeptide is complexed with a lipid, as represented by claim 26 and that finds support in the specification at least in the original claims and at paragraph **[0208]**, for example.

In specific cases of the invention, the method is further defined as a method of preventing growth of a cell in an individual, as represented by claim 41 and that finds support in the specification at least in the original claims and at paragraph **[0029]**, for example. In certain embodiments, the method is further defined as comprising modifying the Bik polypeptide at amino acid position 33, amino acid position 35, or both, wherein the modification results in an inability of the amino acid to be phosphorylated, as represented by claim 42 and that finds support in the specification at least in the original claims and at paragraphs **[0020]** and **[0022]**, for example.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 12, 14-16, 18-26, and 41-42 are rejected under 35 USC § 112, first paragraph, as failing to comply with the written description requirement.

Claims 12, 14-16, 18-26, and 41-42 are rejected under 35 USC § 112, first paragraph, as failing to comply with the enablement requirement.

VII. ARGUMENT

A. Substantial Evidence Required to Uphold the Examiner's Position

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Issues under 35 U.S.C. § 112, first paragraph-Written Description

1. Legal Standard for Written Description

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991). There is no *in haec verba* requirement for written description. In particular, the Federal Circuit has stated that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed.'" *Union Oil Co. of California v. Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ 2d 1227, 1232 (Fed. Cir. 2000). The Federal Circuit has also noted that " [if] a person of ordinary skill in the art would have understood the inventor to have been in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate written description requirement is met." *In re Alton*, 76 F.3d 1168, 1175, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996).

In rejecting a claim under the written description requirement, the Examiner has the initial burden of presenting evidence or reasons why a person skilled in the art would not recognize in an Applicant's disclosure a description of the invention defined in the claims. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976). Accordingly, the Examiner is required: (1) to set forth the claim limitation not described; and (2) to provide reasons why a person skilled in the art would not have recognized the description of the limitation in view of the disclosure of the application as filed. *Interim Guidelines for the*

Examination of Patent Applications Under 35 U.S.C. 112, Paragraph 1, Chisum on Patents, vol. 3, §7.04[1][c].

The Guidelines state that the "written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus."

2. Claims are Supported

Claims 12, 14-16, 18-26, and 41-42 were rejected under 35 U.S.C. § 112, first paragraph, for failing to comply with the written description requirement by allegedly claiming subject matter that was not described in the specification in such a way as to reasonably convey to the skilled artisan that the inventor had possession of the invention at the time of filing.

The Examiner states the following beginning on page 3 of the Action:

> The specification does not disclose **any other mutant Bik polypeptides having an altered amino acid sequence** relative to SEQ ID NO:3 that comprises **at least any amino acid mutation** at position 33 and 35, any mutant Bik having **any altered amino acid sequence in addition to** having Thr^{33} to Asp^{33} and Ser^{35} to Asp^{35} substitutions, or **any mutant Bik polypeptide comprising any modification** at position 33 and 35 that results in an inability of the amino acid to be phosphorylated…

The Examiner further considers that the claims lack "sufficient distinguishing identifying characteristics of the genus," including disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof.

In rejecting a claim under the written description requirement, the Examiner has the initial burden of presenting evidence or reasons why a person skilled in the art would not recognize in an Applicant's disclosure a description of the invention defined in the claims. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976). Accordingly, the Examiner is required: (1) to set forth the claim limitation not described; and (2) to provide reasons why a person skilled in the art would not have recognized the description of the limitation in view of the disclosure of the application as filed. *Interim Guidelines for the Examination of Patent Applications Under 35 U.S.C. 112, Paragraph 1*, Chisum on Patents, vol. 3, §7.04[1][c].

The Guidelines state that the "written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus."

Apepllants submit that there is adequate written description for all of the mutant Bik molecules encompassed by the claims. Claim 12, for example, recites a method of inducing anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in a subject by administering a mutant Bik polypeptide having alterations relative to SEQ ID NO:3, wherein the polypeptide has anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity and wherein the polypeptide has a mutation at Thr33 and Ser35 (by election of species, as opposed to Thr33 alone or Ser35 alone). Support for claim 12 can be found in the specification as follows:

- the specification discloses the sequence of Bik polypeptide in SEQ ID NO:3

- the specification discloses the exemplary mutant Bik polypeptide in SEQ ID NO:9
- the specification discusses the generation of mutations in Bik (at least paragraphs **[0221]** to **[0226]**)
- the specification provides exemplary codons for the mutation in Table 1; and
- the specification teaches biologically functional equivalents of mutant Bik (at least paragraphs **[0076]** to **[0085]**).

Clearly the specification discloses the relevant, identifying characteristics (*i.e.*, structure or other physical and/or chemical properties) of the claimed invention. Thus, the disclosure is sufficient to show Appellants were in possession of the claimed invention.

Appellants further note that the Examiner appears to be misapplying the written description requirement as set for the in *Regents of the University of California v. Eli Lilly and Co.*, 119 F. 3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997) and *Enzo Biochem. Inc. v. Gen-Probe Inc*, which are cited by the Examiner on page 4 of the Final Office Action.

Eli Lilly requires only that claims to genetic material require recitation of more than a mere function. *Eli Lilly*, 119 F.3d 1559, 1568 ("In claims to genetic material, however, a generic statement such as 'vertebrate insulin cDNA' or 'mammalian insulin cDNA,' without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function."). The claims of the present application, however, recite more than a mere function. Current claim 1, for example, recites that the polypeptide sequence is relative to SEQ ID NO:3 and that there are substitutions at Thr33 and Ser35.

Nevertheless, the Examiner considers the claims as if there is no guidance for their description, which is an inaccurate assessment of the instant specification. For example, in

the pending claims Appellants provide a reference Bik sequence, SEQ ID NO:3, to give the skilled artisan disclosure of exemplary structure, and they also provide functional characteristics by reciting that the mutant Bik polypeptide induces anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity. Appellants also provide exemplary methods of inhibiting proliferation of cancer cells in the specification at least at paragraphs **[0239]-[0245]**, **[0260]-[0263]** and FIGS. 1-5 of the specification using analogous Bik mutant polynucleotides, yet one of skill in the art would clearly recognize that Appellants had possession of the invention at the time of filing when the specification teaches use of mutant Bik polypeptides (at least at paragraphs **[0007]** and **[0279]-[0287]**) for the same purpose.

The Examiner alleges that Appellants have not met the standards of *Enzo* because the claims provide no functional characteristics coupled to structural features. However, this is also inaccurate, given that the claims provide specific mutations at amino acids mutation at Thr33 or Ser35 that allow the polypeptide to induce anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity. The remainder of the mutant Bik polypeptide other than Thr33 or Ser35 finds guidance in the specification in reference sequence SEQ ID NO:3 and in Table 1, which provides codons for all standard amino acids.

The Examiner further alleges that Appellants have not provided a representative number of mutant Bik polypeptides having a mutation at Thr33 or Ser35 because they have only provided one mutant Bik polypeptide (SEQ ID NO:9, which comprises mutations at both Thr33 or Ser35); however, Appellants provide mutant Bik polypeptides SEQ ID NO:7 (mutation at Thr33) and SEQ ID NO:8 (mutation at Ser35), although Appellants recognize these are drawn to non-elected species in this case. Nevertheless, the Examiner ignores that Appellants have provided sufficient guidance to anyone of skill in the field to employ a representative Bik sequence as a reference for other mutations and have shown possession of such an invention by providing routine methods how to generate (at least at paragraphs

[0076] to **[0085]**) and characterize such mutants for the intended activity. Although at least at paragraphs **[0239]-[0257]**, **[0260]-[0263]** and FIGS. 1-5 demonstrate analogous mutant Bik polynucleotide embodiments, one of skill in the art recognizes analogous and routine practices would be employed for mutant Bik polypeptide embodiments.

Therefore, Appellants have met the written description guidelines in the pending claims, and respectfully request reversal of the rejection by the Board.

C. Issues under 35 U.S.C. § 112, first paragraph-Enablement

1. The Legal Standard for Enablement

To be enabling within the meaning of 35 U.S.C. § 112, the application must contain a description sufficient to enable one skilled in the art to make and use the claimed invention without unduly extensive experimentation. *Atlas Powder Co. v. E.I. du Pont De Nemours & Co.*, 750 F.2d 1569, 1576 (Fed. Cir. 1984). Furthermore, it is well settled that the Examiner has the initial burden of producing reasons that substantiate a rejection based on lack of enablement. See, *In re Marzocchi*, 439 F.2d 220, 224 (C.C.P.A. 1971); *In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). The Examiner's burden requires that the Examiner supply a factual basis or scientific principle to reasonably doubt the accuracy of a clear disclosure. *In re Marzocchi*, 439 F.2d at 224.

Enablement under 35 U.S.C. §112 is not precluded by the necessity for some experimentation such as routine screening. "The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art." *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). In *Wands* the court observed that "[t]he test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with

respect to the direction in which the experimentation should proceed . . . ." *Id.*, quoting *In re Jackson*, 217 USPQ 804, 817 (Bd. App. 1982).

2. Undue Experimentation is Not Required

Claims 12, 14-16, 18-26, and 41-42 were rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to show to one of skill in the art how to make and use the invention. In particular, the Examiner considers there to be undue experimentation required to practice the invention in light of the guidance provided in the specification.

The Examiner failed to establish a *prima facie* case for lack of enablement. The present specification contains a description sufficient to enable one skilled in the art to make and use the full scope of the claimed invention without unduly extensive experimentation. Appellants will focus their discussion on claim 12, as it is the broadest of the rejected claims. Claim 12 reads:

12. A method of inducing anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in a subject, comprising administering to the subject a mutant Bik polypeptide having an altered amino acid sequence, relative to SEQ ID NO:3, that comprises a substitution at least at a mutation at Thr33 or Ser35, wherein the mutant Bik polypeptide induces anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in the subject.

As can be seen, claim 12 recites mutations at specific sites in the Bik polypeptide relative to SEQ ID NO:3, which is disclosed in the specification. In addition, the specification discloses that the mutant Bik polypeptides of the scope of the claims induce anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in a subject. As a result of guidance provided in the specification, the state of the art, and the level of skill in the art, no more than routine screening would be required to practice the full scope of the claimed invention. Enablement under 35 U.S.C. §112 is not precluded by the necessity for

some experimentation such as routine screening. *In re Wands*, 858 F.2d 731, 736-37 (Fed. Cir. 1988).

Methods of mutagenesis are well known to those of skill in the art. For example, site-directed mutagenesis is a technique that is useful for creating specific mutations in a nucleic acid sequence. Methods of performing site-directed mutagenesis are described in the specification at least at paragraphs **[0221]** to **[0226]**). By following the teachings of the specification, one of ordinary skill in the art would be able to, for example, mutate a nucleic acid sequence encoding the Bik polypeptide of SEQ ID NO. 3 to have a mutation in Thr33 or Ser35.

In fact, more specific detail regarding amino acid substitutions is provided in the specification at paragraphs **[0076]** to **[0085].** The specification teaches that amino acid substitutions are generally based on the relative similarity of the amino acid side chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Specification, paragraphs **[0079]** to **[0084]**. Those of ordinary skill in the art would be able to make substitutions that are either more or less conservative based on the type of amino acid side chain (Specification, paragraph **[0079]**, for example). In addition, those of ordinary skill in the art would be able to make substitutions that are either more or less conservative based on the hydropathic index of the amino acids, which is provided in the specification at paragraph **[0080]**.

Furthermore, on page 11 of the Action the Examiner contends that the specification does not provide guidance how to administer a mutant Bik polypeptide to a subject that induces anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity as claimed. The Examiner notes that the specification provides guidance on administering a mutant Bik polypeptide "through transfection of cells (p. 75) or lipid delivery to mice (p. 11). In fact, Appellants' specification teaches at least two different routes of administration of

mutant Bik polypeptides: 1) in liposomes (paragraphs **[0028]** and **[0065]** of the instant specification); or 2) with protein transduction domains, including teaching two exemplary protein transduction domains that may be utilized (HIV Tat or penetratin) (paragraphs **[0028]** and **[0065]** of the instant specification). Appellants further disclose use of a pharmaceutically acceptable excipient for the polypeptides in paragraph **[0030]**. This goes beyond what one of skill in the art would require for guidance, given that one of skill in the art is aware of such routine methods and well-skilled in practicing them. Furthermore, Appellants provide exemplary description of delivery of Bik proteinaceous compositions in at least paragraphs **[0279]** to **[0287]**.

The Examiner also considers that the following issues for the mutant Bik polypeptide are pertinent and not addressed by the instant specification: 1) internalization of the mutant Bik by a cell to initiate apoptosis; 2) targeting of the protein to tumor cells specifically; 3) development of an immune response to the unnaturally-occurring protein; and 4) proper folding of the protein. However, the Examiner uses the Azar, Mathai, and Bowie references (pages 11-13 of the Action) as if the skilled artisan were not already aware of such issues, yet the effectiveness of the composition in relation to its cellular targeting and subcellular localization, immunogenicity, and folding issues, are all routine considerations to one of skill in the art. Despite this, Appellants did recognize tissue specificity for cancer therapeutics in paragraphs **[0264]** to **[0278]** of the specification, and although these embodiments concern mutant Bik polynucleotides, such disclosure teaches the skilled artisan to be aware of this matter.

The courts have determined that it is not required to disclose well-known techniques or scientific principles to those of skill in the art. *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987);

and *Lindemann Maschinenfabrik GMBC v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 U.S.P.Q. 481, 489 (Fed. Cir. 1984). Therefore, it was not necessary for Appellants to disclose the routine concerns of cellular targeting and subcellular localization, immunogenicity, and proper folding.

Although experimentation may be utilized in at least some instances, Appellants agree with the Examiner and *In re Wands* (858 F.2d 731, 8 USPQ2nd 1400 (Fed. Cir. 1988)) that the issue is not whether or not the experimentation is required but is it undue (page 9 of the Action). However, the courts have determined that experimentation may be considerable in quantity if it is routine. It is certainly routine in the art of to employ standard methods to characterize drug compositions, even though these methods may be considered lengthy. As has been determined by the courts, the scope of the enablement must only bear a "reasonable correlation" to the scope of the claims. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). Even if experiments are necessary for mutant Bik polypeptides, a considerable amount of routine experimentation is permissible, especially where the Appellants' specification provides a reasonable amount of guidance with respect to the direction in which experimentation should proceed. *Ex parte Forman*, 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986) *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976).

In fact, time-consuming experiments are acceptable if the type of experimentation is standard in the art. An extended period of experimentation for mutant Bik polypeptides may not be undue if the skilled artisan is given sufficient direction or guidance. *In re Colianni,* 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). Yet further, the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Wands*, 858 F.2d 737, 8 USPQ2d 1404 (Fed. Cir. 1985); *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174

(Int'l Trade Comm'n 1983), *aff'd. sub nom. Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). 'The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine" *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)).

Appellants reiterate from the Response filed August 2, 2006, that the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970), and guidance is not necessary to those skilled in the art, particularly when it is well-recognized that the skill in the art of molecular biology is quite high (*Ex parte Forman*, 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986). On page 14 of the Action, the Examiner extrapolates by stating that "if little is known in the prior art about the nature of the invention and the art is unpredictable, the specification would need more detail as how to make and use the invention in order to be enabling." However, Appellants have provided sufficient detail by providing an exemplary mutant Bik polypeptide (SEQ ID NO:9); exemplary administration routes (at least paragraphs **[0028]** and **[0065]**); an exemplary reference sequence for the polypeptide sequence outside of the mutations at Thr33 and Ser35; exemplary codons for particular amino acids for the polypeptide sequence outside of the mutations at Thr33 and Ser35; and description of delivery and treatment protocols for utilizing the mutant Bik polypeptides (at least paragraphs **[0279]-[0287]**).

Therefore, it would not be undue experimentation to make and use the invention, and Appellants respectfully request reversal of the rejections by the Board.

VIII. CONCLUSION

Appellants have provided arguments that overcome the pending rejections. Appellants respectfully submit that the Office Action's conclusions that the claims should be

rejected are unwarranted. It is therefore requested that the Board overturn the Action's rejections.

Dated: May 14, 2007

Respectfully submitted,

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APPENDIX 1

CLAIMS ON APPEAL

12. A method of inducing anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in a subject, comprising administering to the subject a mutant Bik polypeptide having an altered amino acid sequence, relative to SEQ ID NO:3, that comprises a substitution at least at a mutation at Thr33 or Ser35, wherein the mutant Bik polypeptide induces anti-tumor activity, anti-cell proliferation activity, and/or pro-apoptotic activity in the subject.

14. The method of claim 12, wherein the substitution is a Thr^{33} to Asp^{33} substitution.

15. The method of claim 12, wherein the substitution is a Ser^{35} to Asp^{35} substitution.

16. The method of claim 12, wherein the polypeptide further comprises a protein transduction domain.

18. The method of claim 12, wherein the subject is a human.

19. The method of claim 18, wherein the human has a proliferative cell disorder.

20. The method of claim 19, wherein the proliferative cell disorder is cancer.

21. The method of claim 20, wherein the cancer is breast cancer, prostate cancer, ovarian cancer, sarcoma, lung cancer, brain cancer, pancreatic cancer, liver cancer, bladder cancer, gastrointestinal cancer, leukemia, lymphoma, or myeloma.

22. The method of claim 20, wherein the cancer is estrogen receptor positive, is EGF receptor overexpressing, is Her2/neu-overexpressing, is not Her-2/neu-overexpressing, is Akt overexpressing, is angrogen independent, or is androgen dependent.

23. The method of claim 20, wherein the cancer is a solid tumors, such as, for example, sarcoma, lung, brain, pancreatic, liver, bladder, gastrointestinal cancers, or hematologic malignancies, such as leukemia, lymphoma, and myeloma

24. The method of claim 20, wherein the proliferative cell disorder is restenosis.

25. The method of claim 12, wherein the polypeptide is comprised in pharmacologically acceptable excipient.

26. The method of claim 25, wherein the polypeptide is complexed with a lipid.

41. The method of claim 12, further defined as a method of preventing growth of a cell in an individual.

42. The method of claim 12, further defined as comprising modifying the Bik polypeptide at amino acid position 33, amino acid position 35, or both, wherein the modification results in an inability of the amino acid to be phosphorylated.

APPENDIX 2

EVIDENCE APPENDIX

Exhibit 1. Mathai *et al.* (J. Biol. Chem. 280 (25):23829-23836), made of record in Office Action mailed April 10, 2006

Exhibit 2. Azar and Lorberboum-Galski *et al.* (Apoptosis 2000; 5:531-542), made of record in Office Action mailed April 10, 2006

Exhibit 3 Bowie *et al.* (Science, 1990, 247:1306-1310), made of record in Office Action mailed April 10, 2006

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BH3-only BIK Regulates BAX,BAK-dependent Release of Ca^{2+} from Endoplasmic Reticulum Stores and Mitochondrial Apoptosis during Stress-induced Cell Death*

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BIK, a pro-apoptotic BH3-only member of the BCL-2 family, targets the membrane of the endoplasmic reticulum (ER). It is induced in human cells in response to several stress stimuli, including genotoxic stress (radiation, doxorubicin) and overexpression of E1A or p53 but not by ER stress pathways resulting from protein malfolding. BIK initiates an early release of Ca^{2+} from ER upstream of the activation of effector caspases. Release of the mobile ER Ca^{2+} stores in baby mouse kidney cells doubly deficient in BAX and BAK, on the other hand, is resistant to BIK but is sensitive to ectopic BAK. Over-expression of p53 stimulates recruitment of BAK to the ER, and both its recruitment and assembly into higher order structures is inhibited by BIK small interfering RNA. Employing small interfering RNA knockdowns, we also demonstrated that release of ER Ca^{2+} and mitochondrial apoptosis in human epithelial cells requires BIK and that a Ca^{2+}-regulated target, the dynamin-related GTPase DRP1, is involved in p53-induced mitochondrial fission and release of cytochrome *c* to the cytosol. Endogenous cellular BIK, therefore, regulates a BAX,BAK-dependent ER pathway that contributes to mitochondrial apoptosis.

Utilizing a DNA microarray analysis of genes that are stimulated by the oncogenic E1A protein of adenovirus, we previously identified BH3-only BIK as a strong responder in human KB epithelial cells. E1A is a potent inducer of both BIK protein and apoptosis in human epithelial cells, dependent on its ability to up-regulate the levels of p53 (1). Moreover, overexpression of p53 in p53-null lung H1299 cells also induced BIK mRNA and protein with kinetics very similar to the induction of $p21^{WAF1}$, which is a rapid response protein in this pathway. Additionally, BIK is induced in estrogen-dependent MCF7 breast cancer cells in response to inhibition of estrogen signaling (2), and induction of BIK contributes to the apoptotic selection of mature B lymphocytes (3). The fact that BIK is primarily regulated through induction of BIK protein is consistent with studies indicating that BIK is a constitutively active pro-apoptotic protein.

The murine ortholog of BIK, Blk, is largely restricted to hematopoietic and endothelial cells and, in contrast to BIK, is not induced by genotoxic stress (4). Moreover gene deletion had little if any effect on the sensitivity of murine cells to genotoxic stress, and animals developed normally (4). In contrast to most BH3-only proteins in mouse and man, which exhibit a high degree of amino acid sequence identity (5, 6), the human and mouse orthologs of BIK are only 42.5% identical, despite having very similar gene structures (7, 8). Consistent with the findings reported by Coultas *et al.* (4), we also have found no evidence that Blk mRNA or protein is induced by either genotoxic stress or p53 overexpression in a variety of mouse cell lines and primary cell cultures.[1] Remarkably, therefore, murine Blk and human BIK respond differently to stress stimuli. Consistent with the findings that human BIK may contribute to tumor suppression, there is reported evidence that mutation of the *BIK* gene is a frequent feature of B-cell lymphomas (9), and the chromatin locus 22q13.3, which contains *BIK*, exhibits deletions in human breast and colorectal cancers (10). To better understand the contribution of BIK induction to apoptosis in human epithelial cells, we utilized BIK RNA interference.

BH3-only BIK interacts with the multi-BH domain anti-apoptotic members of the BCL-2 family but not with pro-apoptotic BAX and BAK (11–13). It contains a single transmembrane segment at its extreme COOH terminus, but in contrast to most BH3-only proteins, which target mitochondria, BIK is integrated almost exclusively in the membrane of the endoplasmic reticulum (ER)[2] (1, 14). Although other members of the BCL-2 family, including anti-apoptotic BCL-2 itself and the multidomain BAX and BAK pro-apoptotic effector molecules, also target the ER (reviewed in Ref. 33), the role of the ER in supporting the mitochondrial apoptosis pathway is only now beginning to emerge (15–17). In the Fas death pathway, for example, cleavage of BAP31 at the ER membrane causes an early release of ER Ca^{2-} stores and concomitant uptake of Ca^{2+} by mitochondria, which triggers the recruitment of a dynamin-related GTPase, DRP1, to the organelle surface followed by mitochondrial fission (18). DRP1 is responsible for scission of the outer membrane during normal mitochondrial fission and fusion, and in the absence of fusion, converts the tubular "worm-like" network of steady-state mitochondria into punctiform fragments (19, 20). Such DRP1-dependent mitochondrial fragmentation is an early event in several apoptotic pathways (21), and in these pathways, DRP1 appears to be necessary for effective egress of cytochrome *c* from the organelle to the cytosol (15, 21). Cytoplasmic cytochrome *c*, in

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[1] J. P. Mathai, M. Germain, and G. C. Shore, unpublished data.

[2] The abbreviations used are: ER, endoplasmic reticulum; siRNA, small interfering ribonucleic acid; HA, hemagglutinin; LM, light membrane; rtTa, reverse tet transactivating protein; RNAi, RNA interference; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; DKO, double knock-out.

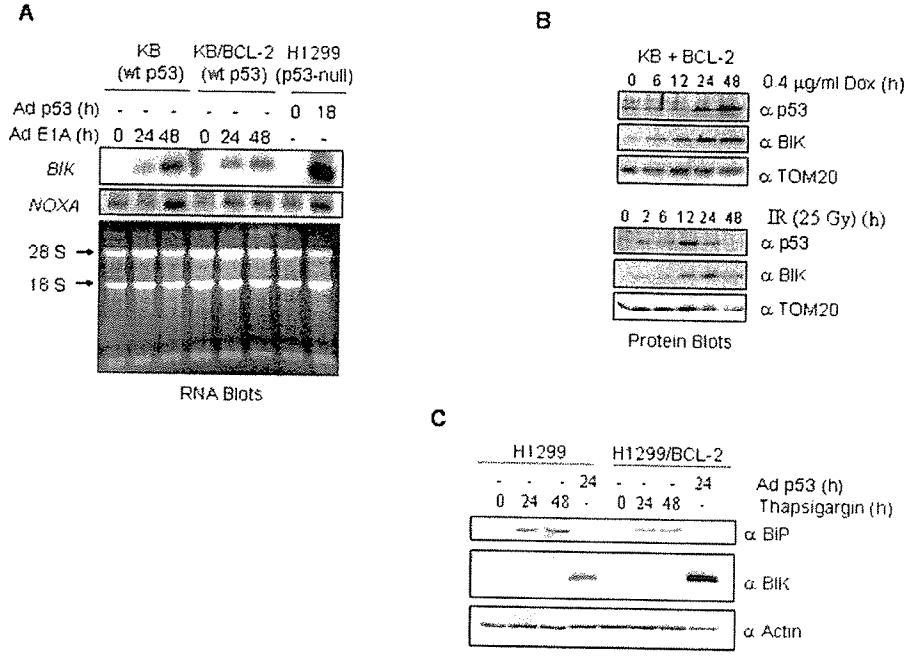

FIG. 1. **Induction of BIK mRNA and protein expression.** *A*, BIK is induced by the oncogene E1A. H1299 lung carcinoma and KB oral epithelial cell lines stably expressing or not expressing HA-BCL-2 were infected for the indicated periods of time with either Ad p53 or Ad E1A vectors. Expression of the indicated genes was determined by Northern blot analysis using corresponding cDNA probes (see "Experimental Procedures"). The bands corresponding to 26 and 18 S ribosomal RNA are indicated and provide gel loading controls. *B*, activation of endogenous p53 results in BIK expression. KB cell lines expressing HA-BCL-2 were exposed to 25 gray of γ radiation or treated with 0.4 μg/ml doxorubicin (*Dox*). The cells were harvested at the indicated times and BIK expression analyzed by Western blot. *C*, ER stress does not induce BIK expression. Protein levels of binding protein, BIK, and actin from H1299 cells lines stably expressing HA-BCL-2, treated with either 2 μM thapsigargin or infected with Ad p53 for the indicated times. *Gy*, gray; *wt*, wild type; *BiP*, binding protein.

turn, becomes a critical constituent of the apoptosome, which processes and activates effector procaspases (22, 23).

Recent work has established that BAX,BAK regulates ER Ca^{2+} homeostasis (24, 25). Here we employed an adenovirus containing p53 as a tool to induce endogenous BIK in p53-null H1299 human lung epithelial cells. Utilizing BIK siRNAs, we demonstrated that BIK induction in this system is required to initiate early release of Ca^{2+} from the ER, mitochondrial fragmentation, and activation of the mitochondrial cytochrome *c* release pathway. BIK initiates the release of Ca^{2+} from ER stores by a pathway dependent on BAX,BAK.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Reagents—Stable human KB oral epithelial and H1299 lung carcinoma cell lines, either expressing or not expressing ectopic HA-BCL-2 (26), were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and 100 mg/ml streptomycin and penicillin. Transformed baby mouse kidney epithelial cells and baby mouse kidney cells derived from *BAX,BAK* doubly deficient (DKO) mice (36) were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and 100 mg/ml streptomycin and penicillin.

Plasmids encoding CFP fused to the NH_2 terminus of DRP1(K38E) were gifts from H. McBride (Ottawa Heart institute, Ottawa, Ontario, Canada). pGL3-CMV and pRL-CMV plasmids were from Promega. Carbobenzoxy-valvy-alanyl-aspartylmethyl ester-fluoromethyl ketone (Z-VAD-fmk) was purchased from Enzyme System Products, Fura-2/AM was from Calbiochem, and doxorubicin and thapsigargin were purchased from Sigma. All transfections were performed using Lipofectamine™ Plus (Invitrogen) according to the manufacturer's protocols.

Cloning of NOXA cDNA and Northern Blots—NOXA cDNA was cloned as described in Ref. 1 utilizing the sequences deposited in GenBank™ (accession number D90070). The primers used for the cloning of NOXA were 5′-TTGGATCCCTCCAGTTGGAGGCTGAGGTT-3′ and 5′-CGGAATTCCTTGAAGGAGTCCCCTCATGC-3′. Northern blots were performed as described in Ref. 1 using 30 μg of total RNA extracted from H1299 cells or KB cells, either expressing or not expressing ectopic HA-BCL-2.

RNA Interference of BIK and Viral Infection—The following siRNA duplexes, with a 3′-end dTT overhang and corresponding to two separate regions within the BIK RNA sequence, were purchased from Dharmacon Research (Lafayette, CO) (numbers are in relation to the start site nucleotide for translation): siRNA BIK145, 5′-AUGCAUGGAGGGCAGUGAC-3′; siRNA BIK315 5′-GUUUCAUGGACGGUUUCAC-3′. Double-stranded siRNA duplex 5′-CUUACGCUGAGUACUUCGA-3′ with a 3′-end dTT overhang corresponding to a region within the luciferase gene of the pGL3 plasmid (designated siRNA-LUC) was also purchased for use as a control. The final concentration of siRNA used/transfection was 60 nM. Adenoviral infection of cells was performed ~12 h after transfection with siRNA as described previously (27), using 100 plaque-forming units/cell of virus.

Antibodies, Immunoblots, Immunofluorescence, and Microscopy—The following antibodies were utilized: goat anti-BIK (Santa-Cruz Biotechnology), mouse anti-actin (ICN Biomedical), rabbit anti-TOM20 (described in Ref. 42), monoclonal anti-p53 (Pharmingen), rabbit anti-calnexin and rabbit anti-binding protein (gift from J. Bergeron), mouse anti-cytochrome *c* (Pharmingen), rabbit anti-BAX (Santa-Cruz Biotechnology), and monoclonal anti-BAK (Oncogene Research Products). SDS-PAGE of whole cell lysates, transfer of proteins to nitrocellulose filters, development of blots with antibodies, and detection by enhanced chemiluminescence have been documented in earlier publications (1, 15). For immunofluorescence, cells were plated onto coverslips at ~50% confluency for transfection and adenoviral infection. After the indicated infection times, the cells were treated and visualized as previously described (15). In experiments for Fig. 5*C*, all cells were treated with 5 μM nocodozol for 20 min prior to PFA fixing to aid in the visualization of fission events (28).

Luciferase Assays—The firefly luciferase vector pGL3-CMV was transfected with *Renilla* luciferase vector pRL along with siRNA-LUC using Lipofectamine Plus according to the manufacturer's protocol. The cells well harvested 24 h later and lysates assessed for luciferase activity using a Lumat LB 9507 luminometer and the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instruction.

Ca^{2+} Measurements—Thapsigargin-releasable ER calcium was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM thapsigargin to cells in Ca^{2+}-free buffer (15, 29). In brief, 2×10^6 cells were harvested and washed in Ca^{2+}-free buffer (20 mM HEPES, pH 7.4, 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 0.1% glucose, 0.1% bovine serum albumin, 250 mM sulfipyrazone). The cells were resuspended in 200 μl of calcium-free buffer containing 0.02% pluronic acid and subsequently loaded with the cell-permeable fluorescent indicator Fura-2/AM at 3 mM for 30 min at 37 C. After a final wash, the cells were resuspended in Ca^{2+}-free buffer and a 340/380-nm excitation ratio at a 510-nm emission wavelength were obtained using a LS 50B PerkinElmer Life Sciences luminescence spectrophotometer. For Fig. 6, the cells were grown and treated on poly-L-lysine-treated coverslips and loaded by adding 100 μM Fura-2/AM to the culture medium for 30 min. Coverslips were washed with Hanks' buffer (3 mM Na2HPO$_4$, 5.4 mM KCl, 0.4 mM KH2PO$_4$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.8 mM $MgSO_4$, 5 mM HEPES, 10 mM glucose, 137 mM NaCl, 4.2 mM $NaHCO_3$) followed by two washes with Ca^{2+}-free Hanks' Buffer (3 mM Na_2HPO_4, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.68 mM NaCl, 0.5 mM $MgCl_2$, 0.8 mM $MgSO_4$, 5 mM HEPES, 10 mM glucose, 137 mM NaCl, 4.2 mM $NaHCO_3$). Images were obtained as previously described (30) using an intensified charge-coupled device camera (IC200) and PTI (Photon Technology International Inc., Princeton, NJ) software at a single emission wavelength (510 nm) with a double excitatory wavelength (340 and

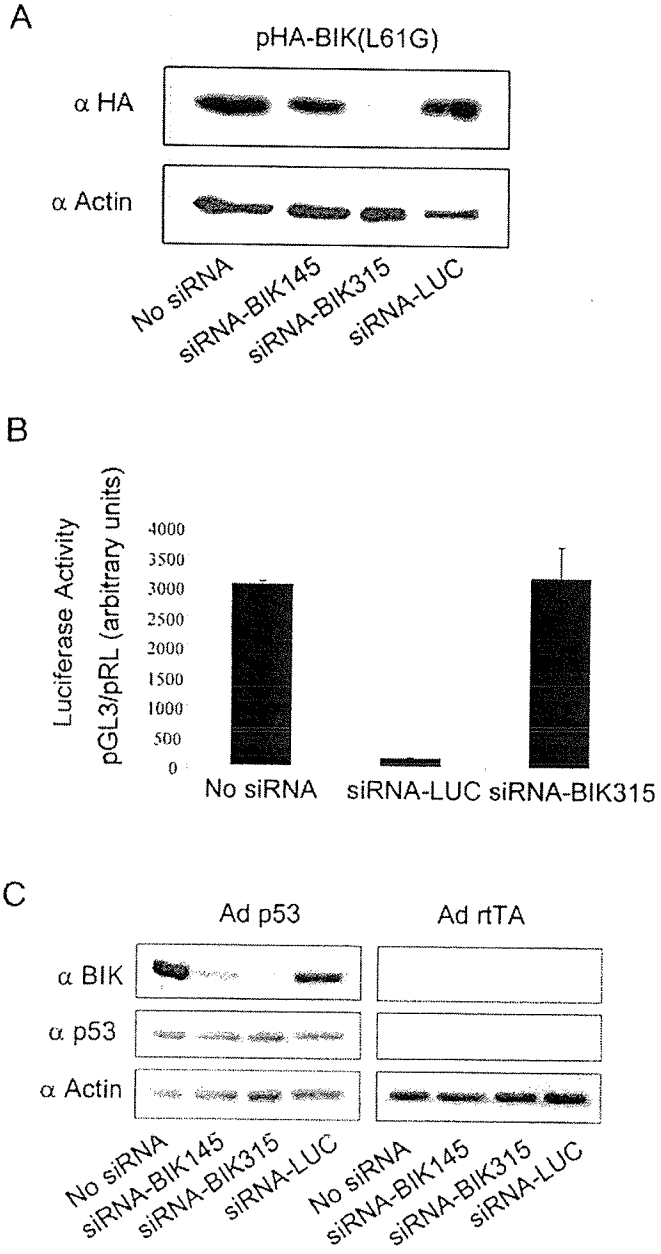

FIG. 2. **siRNA-BIK315 specifically inhibits BIK expression.** *A*, H1299 cells were transfected with an expression plasmid containing HA-BIK L61G along with siRNAs as indicated. Cells were harvested after 24 h and total cell lysates analyzed by SDS-PAGE and immunoblotting. *B*, the plasmids pGL3-CMV and pRL-CMV (internal control) containing different luciferase reporter genes were co-transfected into H1299 cells along with siRNA-LUC or siRNA-BIK315. The cells were collected after 24 h and luciferase activity measured. Shown are mean ± S.D. of three independent experiments. *C*, H1299 cells were transfected with the indicated siRNAs and infected with either Ad p53 or control Ad rtTa. The cell lysates were collected and analyzed for BIK expression by SDS-PAGE and immunoblotting using the indicated antibodies. *LUC*, luciferase.

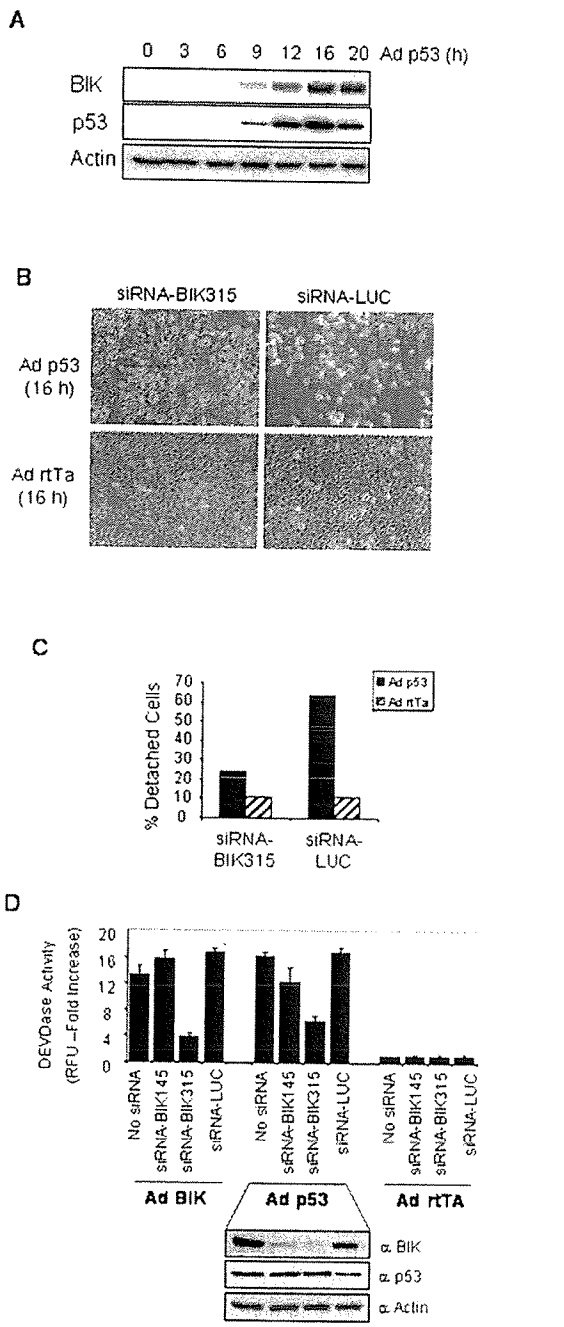

FIG. 3. **BIK knockdown prevents p53-induced morphological changes and caspase activation.** *A*, time course of BIK induction by p53. H1299 cells were infected with Ad p53 for the indicated times, and the expression of BIK and p53 protein were assessed by Western blot analysis of cell lysates. *B*, H1299 cells were transfected with either siRNA-BIK315 or siRNA-LUC, followed by infection with Ad p53 or control Ad rtTa for 16 h. Cells were visualized by phase contrast light microscopy. *C*, the detached cells from the culture medium in *B* were collected and counted. The remaining adherent cells were trypsinized, counted, and the percentage of detached cells from the total was calculated. Shown is a representative of five independent experiments. *D*, as in *B*, caspase-3 like protease activity was measured by the ability of cell lysates to hydrolyze the fluorogenic caspase substrate DEVD-7-amino-4-methylcoumarin. Data presented are means ± S.D. for three independent experiments and are expressed as the fold increase in DEVDase activity compared with mock-transfected rtTa-infected cells. Cell extracts from p53-infected cells were analyzed by Western blotting to assess the extent of BIK knockdown (*gel insert*). *LUC*, luciferase; *RFU*, relative fluorescence units.

380 nm). Fluorescence ratio (340/380) was measured in cells treated with 2 μM thapsigargin and the Fura-2 ratio values converted to $[Ca^{2+}]$ according to the formula of Grynkiewicz *et al.* (31). The peak thapsigargin-releasable $[Ca^{2+}]_{cyto}$ was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM thapsigargin to cells in Ca^{2+}-free Hanks' buffer.

RESULTS AND DISCUSSION

BIK Expression Is Induced by Oncogenic and Genotoxic but Not ER Stress—We have previously shown that BIK is induced by adenovirus E1A in a p53-dependent manner. The resulting BIK protein accumulates to especially high levels in cells expressing BCL-2, because BIK is induced upstream of BCL-2 and does not decay in these BCL-2-protected cells (1). In Fig.

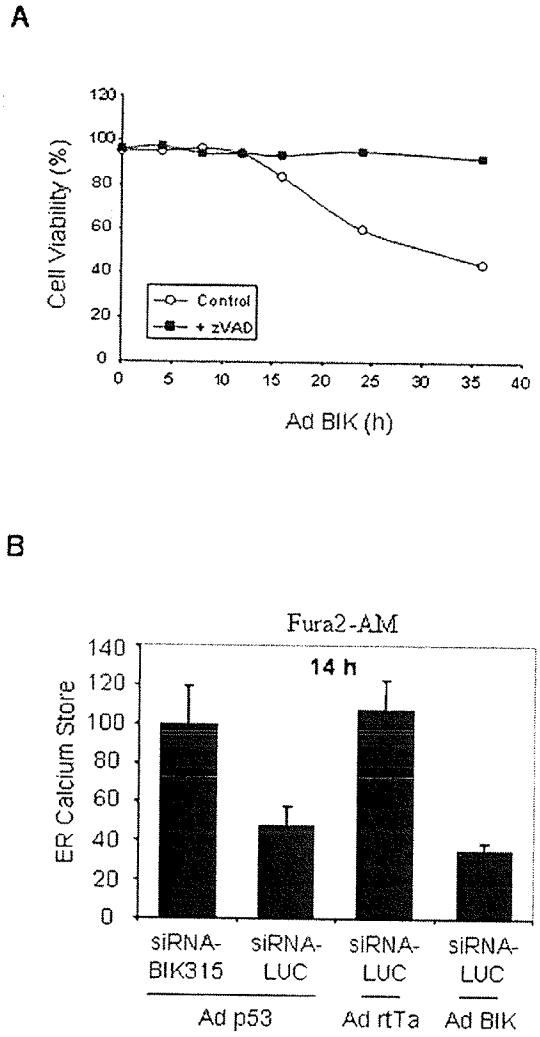

FIG. 4. **p53-regulated ER calcium sensitivity is diminished by BIK knockdown.** *A*, H1299 cells were infected with Ad BIK in the presence (*open circle*) or absence (*closed square*) of 50 μM Z-VAD-fmk, and cell viability was measured as the percentage of cells that excluded trypan blue at the indicated times. *B*, p53-induced ER calcium release is reduced by BIK knockdown. H1299 cells were transfected with siRNA-BIK315 or siRNA-LUC, followed by infection with either Ad p53, Ad BIK, or control Ad rtTa for 14 h. The cells were then loaded with Fura-2/AM, and peak cytosolic Ca^{2+} concentrations were measured as the difference in Fura-2 fluorescence recorded before and after the addition of 2 μM thapsigargin. Data is presented relative to that of untreated cells. Shown are the mean ± S.D. of five independent experiments. *LUC*, luciferase.

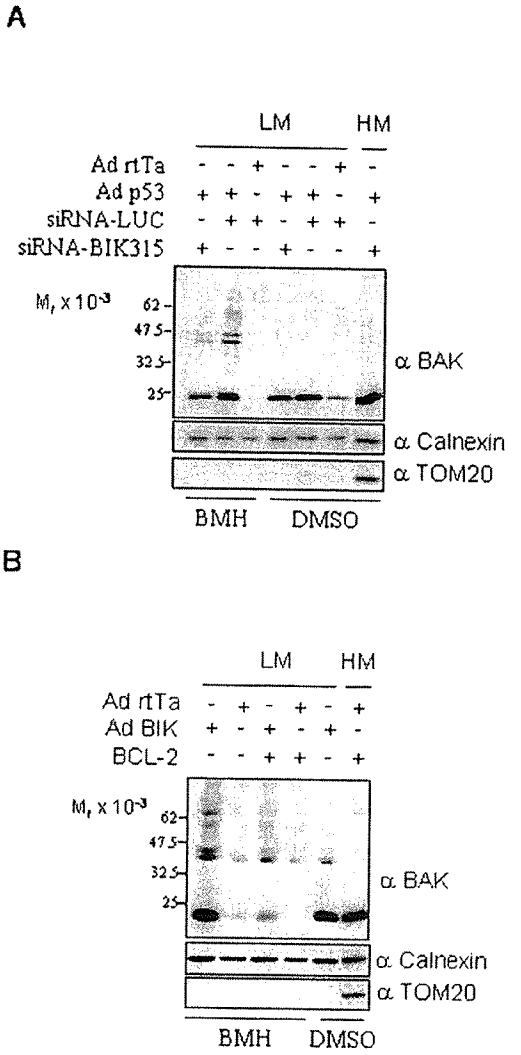

FIG. 5. **BIK promotes BAK localization and oligomerization at the ER.** *A*, p53 induces ER BAK localization and oligomerization diminished by siRNA-BIK315. H1299 cells were transfected with siRNA-BIK315 or siRNA-LUC and infected with either Ad p53 or Ad rtTa in the presence of Z-VAD-fmk. Light membrane (*LM*) and mitochondrial fractions were isolated 13 h after infection, treated with 0.5 mM bismaleimidohexane (*BMH*) or Me_2SO, (*DMSO*), and the fractions analyzed by SDS-PAGE and probed with the indicated antibodies. *B*, BIK induces BAK ER localization and oligomerization attenuated by BCL-2. H1299 cells or H1299 stably expressing BCL-2 were infected with either Ad-BIK or Ad-rtTa in the presence of 50 μM Z-VAD-fmk for 13 h. ER and mitochondrial fractions were isolated and treated as in *A*. *HM*, heavy membrane.

1*A*, expression of BIK mRNA, together with that of another p53-inducible gene product, NOXA, was assessed by Northern blots of total RNA following infection of KB epithelial cells (p53 wild type) with Ad E1A, which elicits a strong pro-apoptotic stress stimulus. The adenoviral vector Ad5 dl520E1B was used for this purpose (32), which delivers only the pro-apoptotic 243-amino acid E1A12S oncoprotein, with no E1B products, which are protective against cell death agonists. Pro-apoptotic cell stress can also be initiated by overexpressing p53 itself in p53-null cells (1). For reference, the p53−/− human lung carcinoma cell line H1299 was infected with an adenoviral vector encoding wild-type human p53 (Ad p53). BIK mRNA was undetectable prior to delivery of Ad E1A or Ad p53 (time 0, Fig. 1*A*). The subsequent increase of *BIK* mRNA in response to these inducers, however, was robust. In contrast to BIK protein levels (1), BCL-2 did not strongly influence BIK mRNA levels. Because BIK protein is induced by E1A in a p53-dependent manner (1), we also examined stimuli that up-regulate endogenous p53 in KB cells. As shown in Fig. 1*B*, genotoxic damage conferred by exposure of the cells to 25 gray of γ radiation or treatment with 0.4 μg/ml topoisomerase inhibitor doxorubicin also stimulated BIK protein induction in parallel with the accumulation of p53. Because BIK is strongly concentrated at the ER from where it is able to exert its pro-apoptotic function independent of a mitochondrial association (1, 14, 33), we also sought to determine whether BIK induction might occur in response to ER stress stimuli. To that end, we treated H1299 cells overexpressing BCL-2 with either Ad p53 or the ER stressor thapsigargin for the indicated times. Thapsigargin inhibits the sarcoplasmic/endoplasmic reticulum calcium ATPase pump, thereby preventing normal Ca^{2+} uptake into the ER from the cytosol and causing depletion of releasable ER Ca^{2+} by passive leak. Over time, this leads to induction of unfolded protein response proteins, such as the chaperone binding protein (Fig. 1*C*) and ultimately apoptosis. Even after 48 h, how-

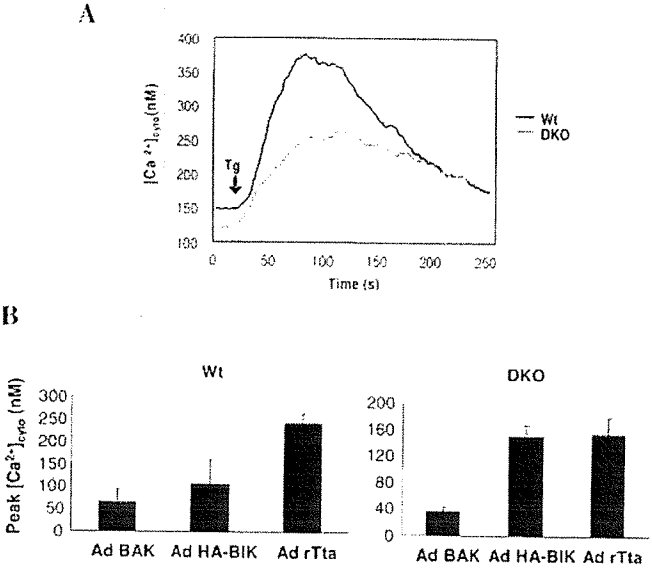

FIG. 6. **BIK-induced ER Ca^{2+} release is dependent on BAX and BAK.** *A*, representative trace of $[Ca^{2+}]_{cyto}$ from Fura-2-loaded wild-type (*Wt*) and DKO cells treated with 2 μM thapsigargin. *B*, wild-type or DKO baby mouse kidney cells were infected with BAK at a multiplicity of infection of the 20 and with BIK or rtTa at a multiplicity of infection of 100 adenoviral vectors for 11, 14, and 15 h, respectively, in the presence of 50 μM Z-VAD-fmk. Peak $[Ca^{2+}]_{cyto}$ of Fura-2-loaded cells was obtained upon the addition of 2 μM thapsigargin. Shown is the mean ± S.D. of three independent experiments measuring 10–20 individual cells/experiment.

ever, no evidence of BIK induction was observed despite the observed induction of binding protein by 24 h. Similar observations were made with another ER stressor, tunicamycin, and with different cell lines. Moreover, the times at which the presence of BIK was examined overlapped with the appearance of dying cells (data not shown). BIK regulation, therefore, is not sensitive to ER stress.

Knockdown of BIK by siRNA—To investigate the role of BIK in situations where it is induced, we employed RNA interference (RNAi). To this end, we designed the small interfering ribonucleic acid (siRNA) duplexes siRNA-BIK145 and siRNA-BIK315, which are homologous to regions within the BIK coding sequence initiating at nucleotides 145 and 315 relative to the start site of translation, respectively. An HA-BIK mutant harboring a disabling point mutation within its BH3 region (L61G), which permits high accumulation of the protein (1), was co-transfected with siRNAs BIK315 or BIK145 or a control siRNA targeting the luciferase gene within the pGL3-CMV vector (designated siRNA-LUC, Fig. 2*A*). siRNA-BIK315 exhibited a strong inhibition of BIK accumulation, whereas siRNA-BIK145 was a weaker inhibitor, and siRNA-LUC showed no effect on BIK expression. The endogenous protein levels of actin were also not significantly affected by any of the siRNA duplexes. To further confirm the specificity of siRNAi-BIK315, the vector pRL-CMV, which encodes the gene for *Renilla* luciferase, was co-transfected with the pGL3-CMV plasmid, which contains the gene for firefly luciferase. siRNA-BIK315 or control siRNA-LUC were also included in the transfection, and the luciferase activity quantified after 24 h. As shown in Fig. 2*B*, the siRNA-LUC inhibited nearly all firefly luciferase activity, whereas siRNA-BIK315 had no effect on activity as compared with the control. Thus siRNA-BIK315 is both a strong and specific inhibitor of BIK protein expression. In Fig. 2*C*, the siRNAs were analyzed for their ability to knock down endogenous BIK in H1299 cells infected with Ad p53. Again, siRNA-BIK315 strongly inhibited Ad p53-induced BIK expression, whereas siRNA-BIK145 also inhibited but to a lesser extent, and siRNA-LUC had no effect. The ability of siRNA-BIK145 to inhibit induction of endogenous BIK (Fig. 2*C*) more effectively than its ability to counter the large amount of BIK(L61G) generated in BIK-transfected cells (Fig. 2*A*) is consistent with siRNA-BIK145 exhibiting intermediate effectiveness against its target. Thus, siRNA-BIK315 serves as an effective means to specifically knockdown expression of endogenous BIK, with siRNA-BIK145 as a potential intermediate inhibitor and siRNA-LUC as a negative control molecule.

BIK Is Required for Activation of Caspases in Response to Ad p53—Fig. 3*A* shows the time course of appearance of BIK and p53 proteins following infection of p53-null H1299 cells with Ad p53; both proteins were detectable by 9 h post-infection. By 16 h of infection with Ad p53, H1299 cells typically exhibit classical changes characteristic of the apoptotic phenotype, such as cell rounding, membrane blebbing, and activation of caspases (1) (Fig. 3*B*). Transfection with siRNA-BIK315 inhibited these p53-induced morphological transformations from occurring at 16 h. In the presence of siRNA-BIK315, Ad p53-infected cells looked similar to those infected with control adenovirus vector encoding reverse tet transactivating protein (Ad rtTa) (Fig. 3*B*), with over three times the number of cells remaining adherent to cell culture plates compared with that of the siRNA-LUC control (Fig. 3*C*). Activation of effector caspases (DEVDase activity) was optimally detected by 16 h post-infection with Ad p53 (not shown). This was also attenuated by knock down by siRNA-BIK315 of both endogenous BIK induced by Ad p53 and ectopic BIK expressed by Ad BIK (Fig. 3*D*). As expected, infection with control Ad rtTa vector did not result in activation of effector caspase activity. Of note, although siRNA-BIK145 was capable of knocking down a significant fraction of the endogenous BIK that was induced by p53 (Fig. 3*D*, *gel insert*), substantial effector caspase activity was still observed, although lower than that of cells transfected with control siRNA-LUC. This is in contrast to cells in which p53-induced BIK expression was nearly completely knocked down by siRNA315 (Fig. 3*D*, *gel insert*), where the corresponding caspase activity was more strongly inhibited. Thus, there is a dose-dependent inhibition of caspase activation in response to the extent of BIK knockdown, which further validates the specificity of the BIK siRNA and confirms that BIK plays an important role in the stress-induced apoptosis elicited by overexpression of p53.

BIK Medites Early Ca^{2+} Release from ER—Emerging evidence suggests that Ca^{2+} signaling by the ER contributes to the mitochondrial apoptosis pathway (15, 21). Because these ER-mediated events occur upstream of activation of effector caspases (15), we focused our analysis at earlier times (14 h post-infection) following infection of cells with Ad p53. Moreover, we included 50 μM Z-VAD-fmk in all subsequent assays, because this inhibitor effectively blocks the activation of caspases (11, 20) and loss of cell viability (Fig. 4*A*) that can result from exposure of cells to BIK over an extended time period.

Consistent with a role for BIK in this ER calcium signaling, we found that infection of H1299 cells with Ad BIK in the presence of Z-VAD-fmk induced early and robust release of Ca^{2+} from ER stores, whereas the control adenovirus vector, Ad rtTa, did not (Fig. 4*B*, *right*). The loss of ER Ca^{2+} was measured by loading cells with the cytosolic Ca^{2+}-sensitive dye Fura-2 in the absence of extracellular Ca^{2+} and determining the difference in peak $[Ca^{2+}]_{cyto}$ before and after the addition of thapsigargin, which causes immediate depletion of ER calcium stores. Similar to Ad BIK, Ad p53 also induced an early loss of ER Ca^{2+} (14 h post-infection) to an extent similar to that seen for Ad BIK, and importantly, this response to Ad p53 was

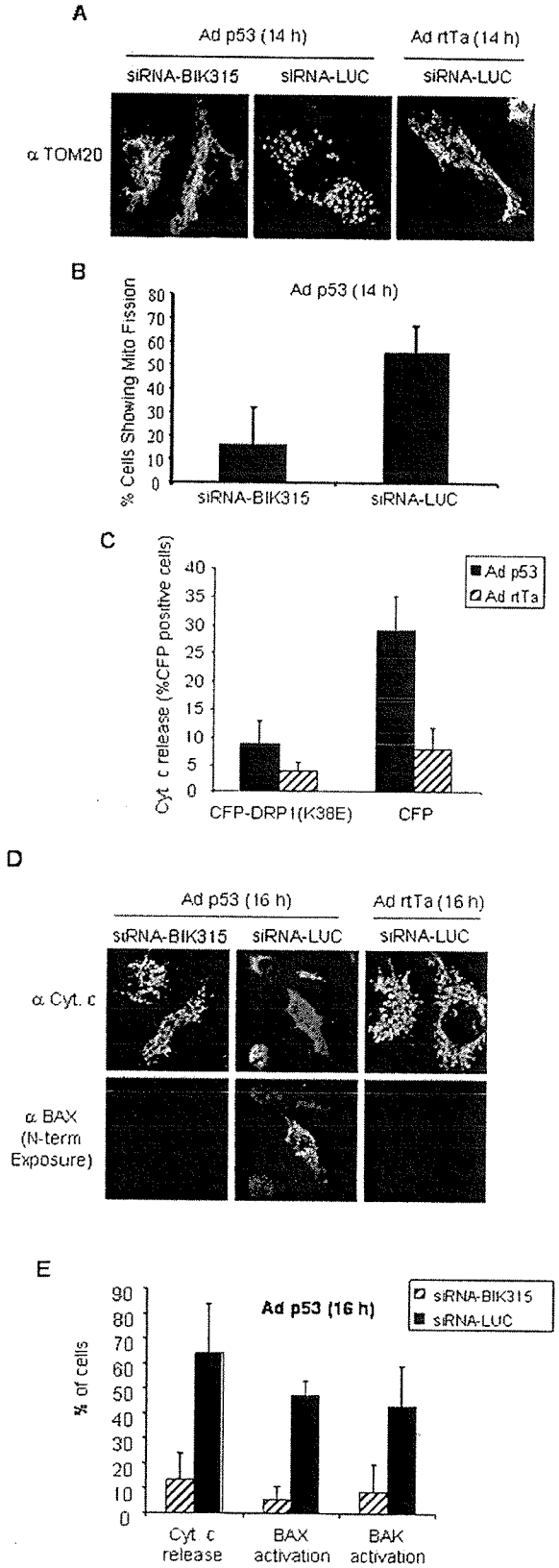

FIG. 7. **BIK knockdown inhibits p53-induced fission of mitochondria, cytochrome *c* release, and BAX/BAK activation.** *A*, BIK knockdown mitigates p53-induced mitochondrial fission. H1299 cells were transfected with either siRNA- BIK315 or siRNA-LUC, followed by infection with Ad p53 or control Ad rtTa for 14 h in the presence of 50 μM Z-VAD-fmk. The cells were then fixed and stained with anti-TOM 20 antibody (representative images are shown). *B*, the percentage of cells from *A* with mitochondrial fission was scored. Shown is the mean ± S.D. of four independent experiments. *C*, CFP-DRP1(K38E) prevents p53-induced cytochrome *c* release. H1299 cells were transiently transfected with CFP or CFP-DRP1(K38E) and subsequently infected with either Ad p53 or control Ad rtTa in the presence of Z-VAD-fmk. 13 h post-infection, the cells were fixed, stained with anti-cytochrome *c* antibody, and immunofluorescence microscopy was used to assess the distribution of cytochrome *c* in cells positive for CFP. Shown is the mean ± S.D. of three independent experiments. *D*, BIK knockdown diminishes p53-induced cytochrome *c* (*Cyt. c*) release and BAX,BAK activation. Transfection was done as in *A*, except the coverslips were fixed at 16 h after infection with Ad p53 or control Ad rtTa and stained with either the anti-cytochrome *c* antibody or active conformation-specific anti-BAX or anti-BAK (not shown) antibodies. Representative images are shown. *E*, the cells in *D* were scored for BAX and BAK activation, as well as cytochrome *c* release. Shown is the mean ± S.D. of three independent experiments.

strongly inhibited by siRNA-BIK315 (Fig. 4*B*, *left*). As expected (14), the pancaspase inhibitor Z-VAD-fmk (50 μM) was without effect on either BIK- or p53-induced release of ER Ca^{2+}, suggesting that Ca^{2+} release from ER in response to Ad p53 was upstream of effector caspases.

BIK Triggers Recruitment and Oligomerization of BAK at the ER—Recent evidence has indicated that, in addition to targeting mitochondria, a relatively small fraction of total cellular BAX and BAK can also reside at the ER, where they undergo oligomerization in response to stress stimuli (25, 34, 35). It has been further suggested that BAK and BAX regulate ER Ca^{2-} stores and, through this mechanism, influence multiple apoptotic signals (25). Because oligomerization of BAK and BAX typically involves BH3-only proteins, we investigated whether BIK might influence BAK oligomerization at the ER. H1299 cells were transfected with siRNA-BIK315 or siRNA-LUC followed by infection with Ad p53 or control Ad rtTa for 13 h in the presence of 50 μM Z-VAD-fmk. Light membranes (LM, enriched in ER membranes) and heavy membranes (enriched in mitochondria) were collected and incubated with the sulfhydryl-reactive chemical cross-linking agent bismaleimidohexane to cross-link oligomerized proteins. In the absence of cross-linker, we observed a strong recruitment of endogenous BAK to the LM after infection with Ad p53 (Fig. 5*A*). Moreover, bismaleimidohexane treatment of LM resulted in the appearance of higher order BAK oligomers following p53 expression (Fig. 5*A*). The LM was not contaminated with mitochondria, as indicated by the absence of the mitochondrial outer membrane-resident protein TOM 20. In Fig. 5, the exposure time of the blots was selected to optimize BAK resolution; in fact, the amount of BAK that distributes to the LM following p53 stimulation is small (10–15%) relative to the pool that is in the heavy membrane fraction. Of note, the ability of Ad p53 to induce localization and oligomerization of BAK at the ER were retarded by siRNA-BIK315 compared with control siRNA-LUC (Fig. 5*A*, *lanes 1* and *2*). To examine the ability of BIK on its own to initiate these events, control H1299 cells or H1299 cells stably overexpressing BCL-2 (1) were infected for 12 h with an Ad BIK or control Ad rtTa, in the presence of 50 μM Z-VAD-fmk. Fig. 5*B* shows that, similar to Ad p53, Ad BIK was also able to trigger BAK ER recruitment and oligomerization. As expected for a BH3-only protein, these BIK-induced events were inhibited by the overexpression of BCL-2.

Effects of BAX,BAK Gene Deletion—The ability of Ad BIK to release mobile stores of Ca^{2+} from the ER was then examined in transformed baby kidney epithelial cells derived from *BAX,BAK* doubly deficient (DKO) mice (36). As previously documented for embryonic fibroblast cells (25), $[Ca^{2+}]_{ER}$ is somewhat lower in DKO epithelial cells compared with wild type (Fig. *A*). Of note, however, strong release of ER Ca^{2+} was observed upon overexpression of ectopic BAK in these DKO cells in the presence of 50 μM Z-VAD-fmk (Fig. 6*B*), indicating that, as in wild-type cells, these ER stores of Ca^{2+} can indeed be mobilized in response to

this pro-apoptotic stimulus. In contrast, DKO cells were strongly resistant to the ability of Ad BIK to stimulate the release of ER Ca^{2+}, whereas wild-type cells were responsive (Fig. 6*B*). Release of ER Ca^{2+} in response to BIK, therefore, is dependent on the pro-apoptotic BAX,BAK setpoint.

Requirement of BIK for Mitochondrial Fragmentation and Release of Cytochrome c to the Cytosol in Response to Ad p53—As previously documented, release of Ca^{2+} from the ER activates a pathway leading to recruitment of DRP1 to tubular mitochondria, causing fragmentation of the organelle and sensitization to stimuli that cause release of cytochrome *c* to the cytosol (18). Fragmentation can be inhibited by a dominant negative active site mutant of the enzyme DRP1(K38E) (20, 28, 37). DRP1(K38E) also inhibits cytochrome *c* release from mitochondria in response to diverse stimuli (15, 21, 38). Thus we examined the influence of CFP-DRP1(K38E)-transfected cells at early stages of Ad p53-induced apoptosis (13 h), compared with control CFP-transfected cells. Again, 50 μM Z-VAD-fmk was included to prevent the potential influence of feedback stimulation by caspases, and the location of cytochrome *c* in Ad p53- or Ad rtTa-infected cells that were marked by CFP expression was determined by immunofluorescence. As shown in Fig. 7*A*, CFP-DRP1(K38E) inhibited the release of cytochrome *c* from mitochondria. Furthermore, H1299 cells treated with siRNA-315 prior to Ad p53 infection retained the extended mitochondrial tubular network that is typically seen in untreated cells. This is in contrast to the fragmented mitochondria seen in those cells transfected with control siRNA (Fig. 7, *A* and *B*), indicating that BIK is required for fission of mitochondria in this pathway. Moreover, BIK knockdown inhibited direct manifestations of mitochondrial apoptosis, including conformational changes associated with activation of mitochondrial BAX/BAK and release of cytochrome *c* to the cytoplasm. This is shown in Fig. 7, *D* and *E*, in which H1299 cells were transfected with siRNA-BIK315 and infected with Ad p53 for 16 h in the presence of 50 μM Z-VAD-fmk. Quantification by immunofluorescence showed a marked decrease in the amount of p53-induced cytochrome *c* release to the cytoplasm compared with control Ad rtTa infection or siRNA-LUC controls (Fig. 7*D*, *top panel*, and Fig. 7*E*, *left*). The use of conformation-specific antibodies, which recognize an exposed NH_2-terminal epitope associated with the active forms of mitochondrial BAX (39, 40) and BAK (41), also revealed that this activation in response to p53 was mitigated by BIK knockdown, because, similar to cytochrome *c* release to the cytosol, these conformational changes were strongly inhibited by siRNA-BIK315 (Fig. 7*D*, *bottom panel*, and Fig. 7*E*, *right*).

In summary, BIK protein is induced in response to select cell stress stimuli, including DNA damaging agents and overexpression of E1A or p53 but not by stress agents that cause protein malfolding in the ER. Of note, however, BIK is located at the ER from where it elicits pro-apoptotic signals and, given sufficient time, these signals lead to cell death by pathway(s) that are strongly inhibited by the wide-spectrum caspase inhibitor Z-VAD-fmk (1, 14). To investigate the initiating events associated with BIK expression, therefore, we focused on the early effects of BIK that still occur in the presence of Z-VAD-fmk, and determined whether these BIK-initiated events contribute to the stress pathway elicited by overexpression of p53 in p53-null human epithelial cells, as judged by BIK knockdown by siRNA. Altogether, the results indicate that BIK induction by Ad p53 is critical for subsequent activation of the mitochondrial apoptosis pathway, with Ca^{2+} release from the ER and DRP1-regulated egress of cytochrome *c* from mitochondria representing early steps in this process. Employing transformed kidney epithelial cells derived from wild-type and *BAX-,BAK* DKO mice, we showed that both cell types maintain a mobile pool of ER Ca^{2+}, because in both cases, significant release of these pools to the cytosol was achieved by expressing ectopic BAK. The fact that BIK, on the other hand, initiated release of ER Ca^{2+} in wild-type but not DKO cells indicates that BIK achieves calcium release through a BAX,BAK-regulated mechanism. As previously documented, such apoptotic release of ER Ca^{2+} contributes to DRP1-dependent fragmentation of mitochondria and release of cytochrome *c* (18), and as shown here, cytochrome *c* egress from mitochondria in response to Ad p53 is also dependent on DRP1. Thus BIK, by initiating BAX,BAK-dependent release of Ca^{2+} from the ER, can contribute to the activation of mitochondrial apoptosis in stress pathways in which BIK protein is induced.

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GnRH-Bik/Bax/Bak chimeric proteins target and kill adenocarcinoma cells; the general use of pro-apoptotic proteins of the Bcl-2 family as novel killing components of targeting chimeric proteins

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In recent years chimeric proteins carrying bacterial toxins as their killing moiety, have been developed to selectively recognize and kill cell populations expressing speciific receptors. The involvement of Gonadotropin releasing hormone (GnRH) has been demonstrated in several adenocarcinomas and a GnRH-bacterial toxin chimeric protein (GnRH-PE66) was thus developed and found to specifically target and kill adenocarcinoma cells both *in vitro* and *in vivo*. Because of the immunogenicity and the non-specific toxicity of the bacterial toxins, we have developed new chimeric proteins, introducing apoptosis inducing proteins of the Bcl-2 family as novel killing components. Sequences encoding the human Bik, Bak or Bax proteins were fused to the GnRH coding sequence at the DNA level and were expressed in E. coli. GnRH-Bik, GnRH-Bak and GnRH-Bax new chimeric proteins efficiently and specifically inhibited the cell growth of adenocarcinoma cell lines and eventually led to cell death. All three Bcl2-proteins-based chimeric proteins seem to induce apoptosis within the target cells, without any additional cell death stimulus. Apoptosis-inducing-proteins of the Bcl-2 family targeted by the GnRH are novel potential therapeutic reagents for adenocarcinoma treatment in humans. This novel approach could be widely applied, using any molecule that binds a specific cell type, fused to an apoptosis-inducing protein.

Keywords: adenocarcinoma cells; apoptosis; Bcl-2 family proteins; chimeric proteins; GnRH-binding site; targeting.

Introduction

The design of specific targeting reagents/drugs still remains the major goal in the treatment of neoplastic diseases. The main aim is to direct therapeutic agents into tumor cells, while avoiding damage to normal tissues and without evoking an immune response.

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In the past few years many chimeric proteins have been developed to target and kill cancer cells. This class of targeting molecules, designed and constructed by gene fusion techniques, comprises both the cell targeting and the cell killing moieties. We recently described GnRH-PE chimeric proteins that can target adenocarcinoma cells, killing them both *in vitro* and *in vivo*.[1,2] In these chimeric proteins, the targeting domain is gonadotroph-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH). The killing moiety is a modified form of the most widely used bacterial toxin, Pseudomonas exotoxin A (PE). GnRH is a hypothalamic decapeptide that participates in the regulation of the reproductive system in humans.[3] Our findings revealed that a wide variety of adenocarcinomas express specific GnRH-binding sites,[1,2] although their identity and functional role remain obscure. GnRH-PE based chimeric proteins can inhibit the *in vitro* growth of some of the major human malignancies such as colon, breast, ovary, lung, kidney and liver adenocarcinomas. Moreover, these GnRH-PE chimeric proteins were able to arrest the growth of established cancer tumors in nude mice (1,2 and our unpublished results). The major obstacle in the clinical application of plant/bacterial-toxin based chimeric proteins is the human immune response they elicit, mainly toward the toxin moiety. Bacterial toxins such as PE are highly immunogenic and cannot be humanized by standard techniques. Moreover, each chimeric protein displays some degree of nonspecific toxicity.[4]

To overcome these disadvantages, we developed a new prototype of chimeric proteins, exploiting apoptosis inducing members of the Bcl-2 family, such as the human Bax protein, as novel killing components. The first prototype chimeric protein, IL2-Bax, specifically targeted cells expressing the IL2 receptor and induced cell-specific apoptosis in the absence of any additional death stimulus.[5] To validate the general use of proteins of the Bcl-2 family as novel human killing moieties in chimeric proteins, we

chose the GnRH binding site as a target and used different pro-apoptotic Bcl-2 proteins, mainly the Bik protein, as well as the Bax and Bak proteins.

The members of the Bcl-2 family of proteins include death agonists (e.g. Bax, Bad, Bik, Bak, Bid and Hrk).[6–9] Except for Bad, whose BH3 domain is within the putative BH1 domain, all of these proteins contain an independent BH3 domain.[10] It appears that only the BH3 domain is required for their pro-apoptotic activity.[9,11] This domain interacts with a hydrophobic cleft formed by the BH1, BH2 and BH3 domains of the anti-apoptotic Bcl-x_L and Bcl-2, as demonstrated in mutational and structural studies.[12]

Mutations within the BH3 domain of Bik also abrogate its ability to induce cell death. Interestingly, Bik, Bid and Hrk, which contain only a BH3 domain, appear to be more potent death effectors than proteins such as Bax and Bak with all three Bcl-2 domains.[7,9] However, the molecular determinants of the "BH3-only" proteins, as opposed to those of the "all BH domains" proteins that trigger the key components of the death pathway(s) leading to activation of the terminal caspases and their function are not clear.

Recently it was suggested that the proapoptotic "BH-3 only" members of the Bcl-2 family induce cytochrome c release, via a different mechanism than that of the Bax protein. BH-3 proteins such as Bik do not induce loss of membrane potential and do not directly modulate voltage-dependent anion channel activity.[13]

Using the BH-3 protein Bik, or other proapoptotic members of the Bcl-2 family (Bax and Bak), as the killing moiety and the GnRH-analog as the targeting component, we now demonstrate that the novel chimeric proteins GnRH-Bik/Bax/Bak specifically target adenocarcinoma cells and induce cell-specific apoptosis.

Materials and methods

Plasmid construction

Total RNA was isolated from fresh human lymphocytes with the TriPure Isolation reagent (Boehringer Mannheim, GmbH, Germany) and then reverse transcribed into first strand cDNA with a reverse transcription system (Promega, Madison, WI, USA). The Bik fragment was generated by PCR, with the synthetic oligonucleotide primers:

5′-GGAATTCAAGCTTTCTCTGAAGTAAGACCC CTCTCC (sense) and 5′-GGGAATTCTCACTTGAGCA GCAGGTGCAG (antisense), which cover the entire coding region. The Bak fragment was generated by PCR, using the synthetic oligonucleotide primers:

5′-GGAATTCAAGCTTTCGCTTCGGGGCAAGGC CCAGGT (sense) and 5′-GGGAATTCTCATGATTTG AAGAATCTTCG (antisense) covering the entire coding region. The PCR reaction mixture was incubated in a DNA thermal cycler (MJ Research Inc. Watertown, MA, USA) for 40 cycles. Each cycle consisted of 1 min. at 95°C, 1 min. at 65°C and 2 min. at 72°C. The PCR fragments were cloned into the pPCR-Script Amp SK+ cloning vector using the PCR-Script Amp Cloning Kit (Stratagene, La Jolla, CA, USA). Using EcoR1 and Hind III, the Bik or Bak inserts were then cut from the pPCR vector, while the entire encoding region of Bax was cut from the pAY1 plasmid.[5] Plasmids encoding GnRH-Bik, GnRH-Bak or GnRH-Bax were constructed by inserting the Bik, Bak or Bax coding sequences flanked by EcoR1 and Hind III restriction sites into the T-GnRH-PE_{66} plasmid, which had been cut with the same endonucleases that remove the full length PE.

Protein expression

E. Coli strain BL21 (λDE3) transformed with a plasmid encoding GnRH-Bik, GnRH-Bak or GnRH-Bax was grown in SLB medium containing 100 μg/ml ampicillin. At $OD_{600} = 1$ the cultures were supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) to induce protein production and then incubated 1.5–2 hours at 37°C. The cultures were spun down the pellet was stored overnight at −70°C and then suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.2 mg/ml lysozyme), sonicated and centrifuged at 35,000 × g for 30 min. The pellet thus obtained was suspended and stirred on ice in denaturation buffer (6 M guanidinium-HCl, 0.1 M Tris-HCl, pH8.6, 1 mM EDTA, 0.05 M NaCl and 10 mM dithiothreitol). The supernatant was cleared by centrifugation at 35,000× g for 15 min. The protein solution was then diluted in refolding buffer (20 mM Tris-HCl, pH8.0, 1 mM EDTA, 0.25 M NaCl and 5 mM dithiothreitol) dialyzed against refolding buffer, followed by dialysis against PBS.

Cell lines

Colon adenocarcinoma SW48 was kindly provided by Aviva Horowitz (Hadassah Medical Center, Jerusalem, Israel). Renal carcinoma 293, cervix adenocarcinoma HeLa and hepatocarcinoma HepG2 were kindly provided by Ruth Shemer (Hebrew University, Jerusalem, Israel). Placental choriocarcinoma JAR, bladder carcinoma T24A and breast adenocarcinoma MCF-7 were kindly provided by Abraham Hochberg (Hebrew University, Jerusalem, Israel). B cell lymphoma Nalm6 and T cell lymphoma Jurkat were kindly provided by Hanna Ben-Bassat (Hadassah Hospital, Jerusalem, Israel). T cell lymphoma HUT 102 was kindly provided by Tom Waldmann (NIH). Colon adenocarcinoma Colo 205, Bladder carcinoma J82

and rhabdomyosarcoma A204 were obtained from the American Type Culture Collection. All the above cells are of human origin.

Mouse T cell lymphoma 2B4 was kindly provided by Rina Gay (Hebrew University, Jerusalem, Israel). Mouse lung adenocarcinoma D122 was kindly provided by Ali Abdul-Hai (Hadassah Hospital, Jerusalem, Israel).

Cell lines were maintained in flasks and grown in a humidified atmosphere of 5% CO_2 at 37°C. Colo 205, SW48, JAR, MCF-7, Nalm6, Jurkat, HUT102 and 2B4 cells were maintained in RPMI-1640 medium. HeLa, 293, HepG2, J82, A204 and D122 cells were maintained in Dulbaco's modified Eagle's medium. T24A cells were maintained in Dulbeco's modified Eagle's medium, Ham's F-12 (1 : 1). All media were supplemented with 2 mM L-glutamine, 100 units/ml penicillin and 100 μg streptomycin. In addition, HUT102 and 2B4 cells were supplemented with 5 mM Hepes buffer solution, 1 mM sodium pyruvate, $\times 1$ MEM amino acids solution and 5×10^5 β-mercaptoethanol.

The media were supplemented with 10% fetal calf serum (FCS), except for Nalm6 and Jurkat cells, which were grown in 20% FCS. All media and supplements were acquired from Biological Industries (Beit Ha'emek, Israel).

Cell cycle staging by FACS analysis

Cell lines were incubated overnight at 37°C (5×10^5 cells/10 ml medium). Chimeric proteins were then added for a period of 24 h, following which the cells were centrifuged at $450 \times g$ for 6 min., washed with cold PBS, resuspended in 300 μl PBS, fixed with 5 ml methanol at −20°C for 1 hr and centrifuged at $800 \times g$ for 5 min. The cells were then resuspended in 1 ml PBS, incubated 30 min. on ice, centrifuged at $800 \times g$ for 5 min. and resuspended in 0.5 ml PBS supplemented with 0.5 μg RNase and 5 μg propidium iodide. The cells were then FACS analyzed for DNA content as a function of cell number by a FACScan (Becton Dickinson, Immunocytomery System, San Jose. CA, USA), using the LYSYS II program.

Measurement of endogenous Bik protein levels by FACS

Cells ($1–2 \times 10^6$) were suspended in 3%FCS/PBS, centrifuged at $300 \times g$ for 5 min., resuspended in 0.5 ml methanol and incubated for 15 min. at 4°C. The cells were washed twice with 3%FCS/PBS, resuspended in 50 μl of the same buffer and incubated with anti-Bik (Santa Cruz Biotechnology Inc. Santa Cruz, California) at 4°C for 1 hr. The cells were then washed twice, resuspended in 50 μl 3%FCS/PBS and incubated with 5 μl FITC conjugated rabbit anti goat IgG (Jackson Immuno Research Lab. West Grove, Pe, USA) for 30 min. at 4°C. The cells were washed twice, resuspended in 0.5 ml PBS and analyzed by a FACScan.

Monitoring cell growth

Cells were incubated overnight in 24-well plates (5×10^4 cells in 1 ml culture medium). Chimeric proteins were then added and the cultures were harvested and counted, monitoring for viability after 24 h, 48 h or 72 h incubation, following which the cells were harvested and counted.

Results

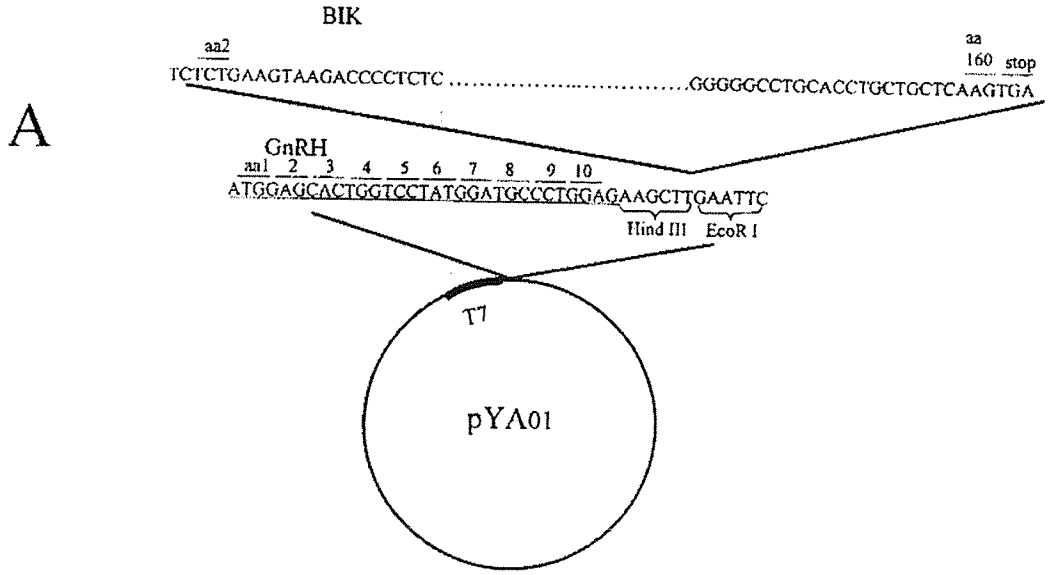
Construction and expression of the plasmids encoding GnRH-Bik/Bax/Bak

A PCR fragment encoding the full length of the human Bik (see Methods) was ligated downstream to a sequence encoding the 10 amino acids of the GnRH analog (tryptophan replacing glycine as the sixth amino acid),[1,2] to generate GnRH-Bik (Figure 1A). Following transformation of E. coli BL21 (λDE3) cells with the plasmid, expression of the fusion gene was controlled by the bacteriophage T7 late promoter.[1,2] GnRH-Bik was found mainly in the insoluble fraction in the form of inclusion bodies, thereby facilitating partial purification of the recombinant protein by denaturation and renaturation of the insoluble fraction.[1,2] Immunoblotting with anti-Bik confirmed the expression of the GnRH-Bik full-length protein (results not shown). The partially purified insoluble fraction (after denaturing and refolding) (Figure 1B) was highly enriched with the GnRH-Bik chimeric protein, enabling us to examine its activity *in vitro*. The two additional chimeric proteins, GnRH-Bax and GnRH-Bak, were constructed, expressed and partially purified in a similar manner.

Effect of GnRH-Bik on target cells

GnRH-bacterial toxin-based chimeric proteins were found to target and kill adenocarcinoma cells.[1,2] Therefore, to examine the activity of GnRH-Bik, we evaluated its effect on two adenocarcinoma cell lines: Colo 205 (colon adenocarcinoma) and 293 (renal adenocarcinoma). This was done by closely monitoring the viability of cultured cells exposed to the chimera for 72 hr. GnRH-Bik efficiently inhibited the cell growth of both cell lines, and eventually led to cell death (Figure 2). GnRH-Bik also reduced the survival of additional adenocarcinoma cell lines originating from breast, renal, cervix and hepatic carcinoma, albeit, with considerable variations between cell lines (Figure 3). The percentage of viable

Figure 1. (**A**) Plasmid pYA1 construct encoding GnRH-Bik. The numbers represent the corresponding amino acids. (**B**) SDS-PAGE analysis of cell fractions containing the GnRH-Bik chimeric protein. 20 μl of the various fractions was run on a 12% SDS-PAGE gel. Lane 1: Whole cell extract of cells expressing GnRH-Bik, Lane 2: Soluble fraction of the expressing cells, Lane 3: Insoluble fraction of the expressing cells. Arrow indicates the GnRH-Bik protein.



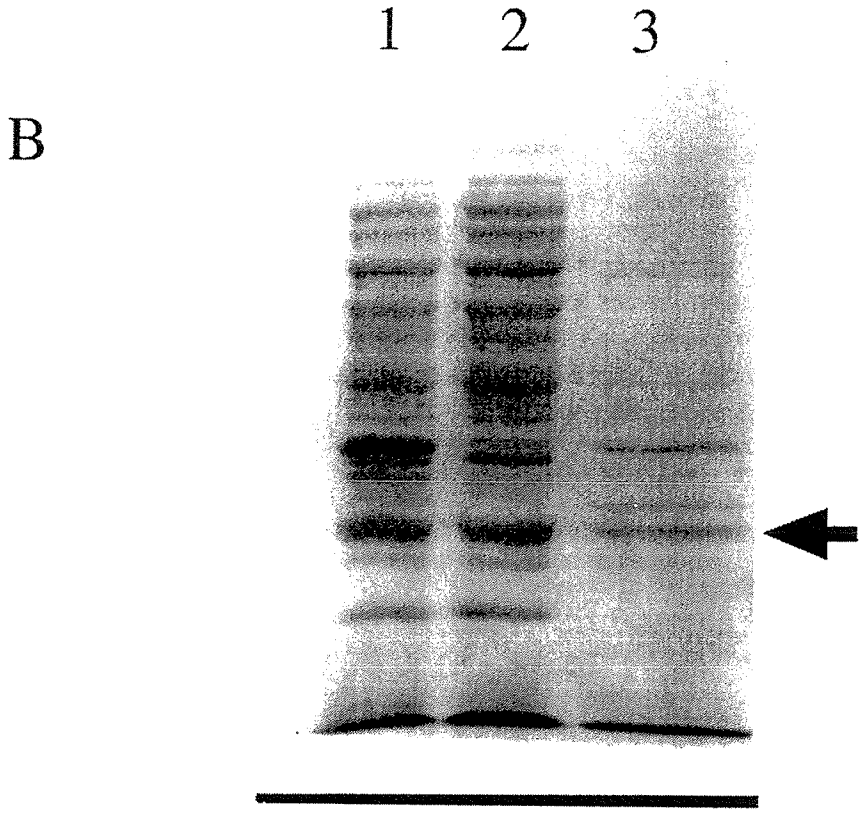
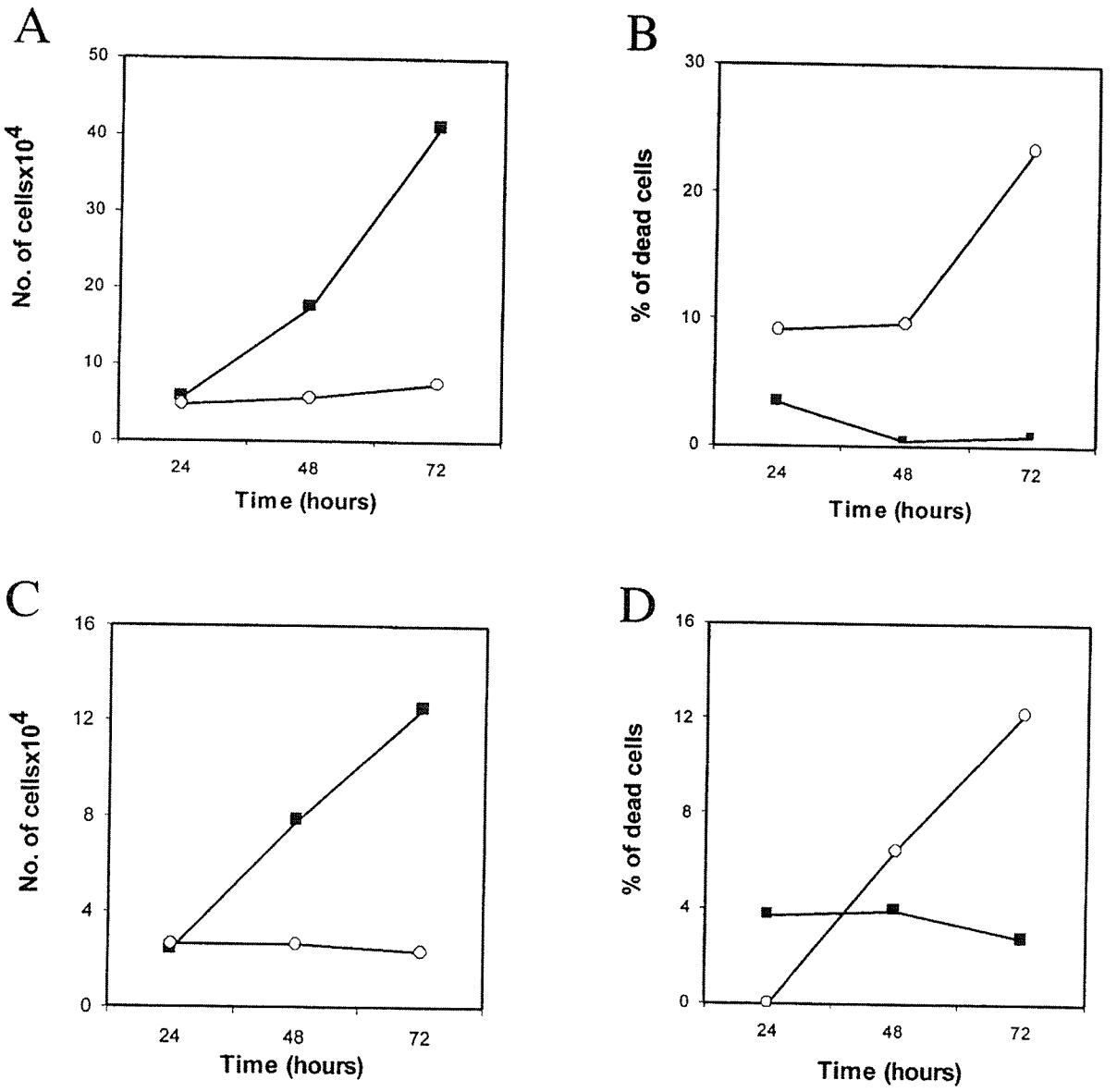


Figure 2. Effect of GnRH-Bik on Colo 205 (A and B) and 293 (C and D) cell growth. A and C: Total cell number in cultures in the presence ○ or absence ■ of GnRH-Bik (5 μg/ml). B and D: Number of dead cells in cultures exposed to GnRH-Bik (5 μg/ml). Cells were stained with Trypan Blue (0.4%).



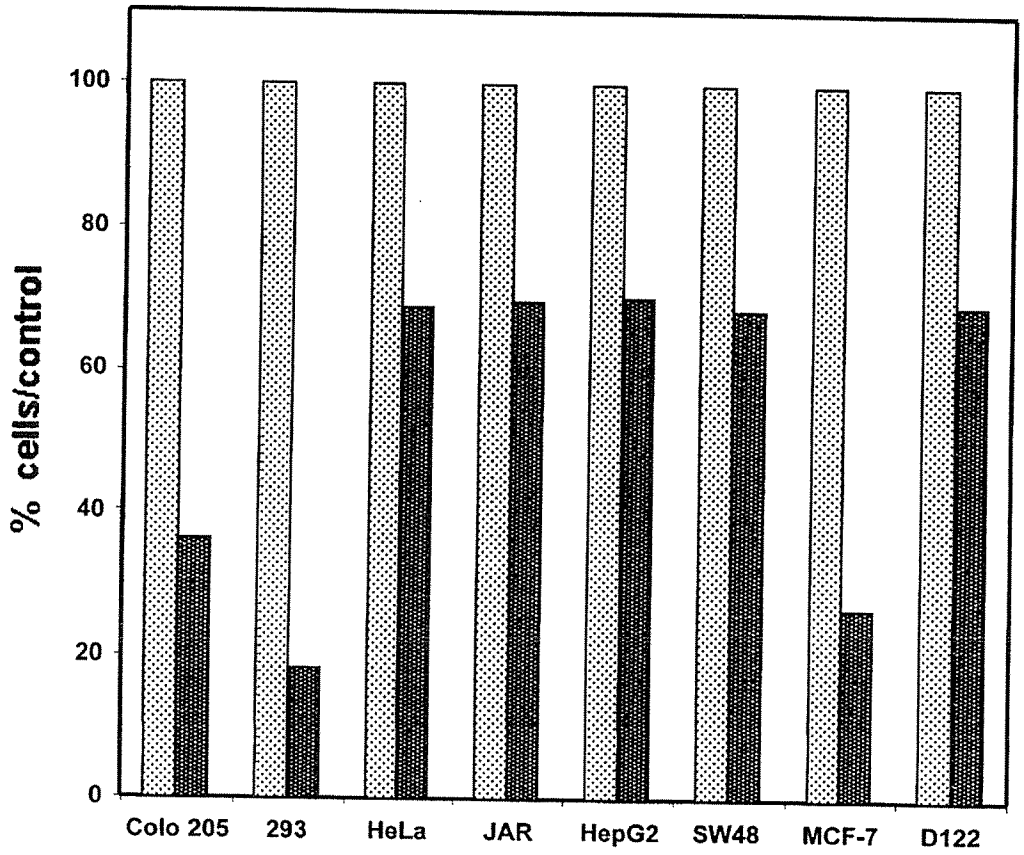
cells remaining in cultures exposed to GnRH-Bik was as low as 18.4% in 293 renal adenocarcinoma and as high as 70% in HepG2 hepatoma cells (Figure 3). GnRH-Bik also inhibited the growth of murine D122 lung adenocarcinoma cells (Figure 3).

GnRH-Bik/Bax/Bak induce apoptosis in target cells

To explore the mechanism, by which GnRH-Bik induces cell death in target cells, we followed by FACS the cell cycle of cultures exposed to the chimera. GnRH-Bik caused an increase in the population of apoptotic cells in Colo 205 cultures in a dose-dependent manner, as assessed by cell cycle staging with PI (Figure 4A). The highest response (80% apoptotic cells) was observed when 10 μg/ml ($\cong 5 \times 10^{-7}$ M of GnRH-Bik) of the chimeric protein were added (Figure 4B). Interestingly, while the apoptotic population of cells increased upon exposure to GnRH-Bik, there was a parallel decrease in the percentage of cells in the S phase (Figure 4B).

The two additional chimeric proteins, GnRH-Bax and GnRH-Bak also caused an increase in the apoptotic

Figure 3. Effect of GnRH-Bik on various adenocarcinoma cell lines. Percentage of viable cells per control cultures in the various cultures in the presence - ■ or absence - ▨ of GnRH-Bik (5 μg/ml). Cells were exposed to the chimera for 72 hr and stained as in Figure 2.



population of Colo 205 cells (Figure 4A and our unpublished results).

Moreover, when the morphology of the cells treated with GnRH-Bik was followed under the microscope, typical apoptotic cells were observed (Figure 5).

Specificity of the GnRH-Bik/Bax/Bak response

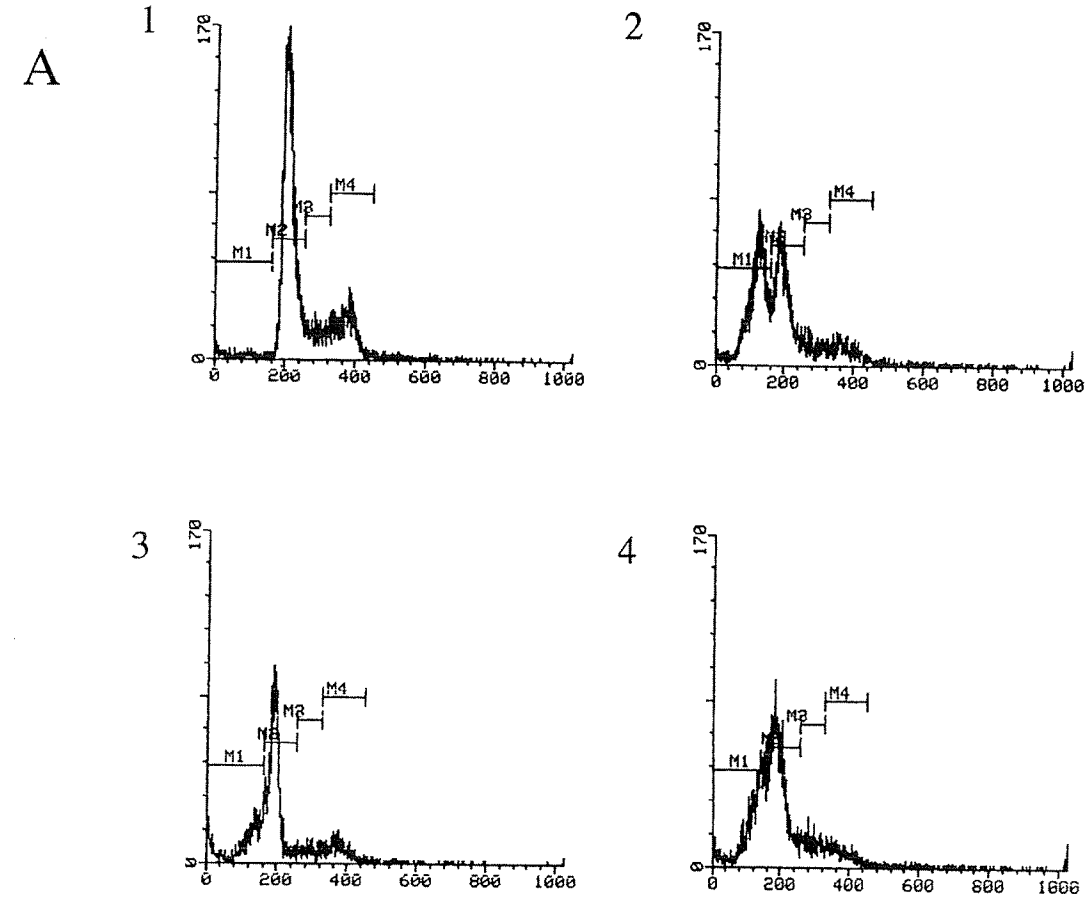
The activity of GnRH-Bik, as assessed by cell count, was tested in the presence of excess GnRH hormone. GnRH strongly inhibited the effect of the GnRH-Bik chimeric protein (Figure 6); thus demonstrating the latter's specific activity. GnRH-Bik had no influence on non-adenocarcinoma cell lines, that do not express the GnRH-binding site, such as bladder carcinoma (T24A and J82), rabdomyosarcoma (A204), B cells (Nalm 6) and T cells (Jurkat, HUT102, 2B4) (Table 1). We also tested control chimeric proteins composed of various irrelevant targeting moieties. None of the control proteins induced the death of the Colo 205 target cells (Figure 5 and our unpublished results). It should be pointed out that in these experiments we used partially purified insoluble fractions (after denaturing and refolding) of the control chimeric proteins, as in the experiments with the GnRH-Bik/Bax/Bak chimeras. GnRH-Bax and GnRH-Bak showed the same specificity, i.e. targeting only adenocarcinoma cells and inducing cell specific apoptosis, similar to the results obtained with GnRH-Bik (results not shown).

Table 1. Effect of GnRH-Bik on survival of non-target cells

		No. of cells × 10^4	
Cell line	Origin	Control	GnRH-Bik (5 μg/ml)
T24A	Bladder carcinoma	18.1 ± 0.5	28.0 ± 8.0
J82	Bladder carcinoma	20.7 ± 1.2	21.0 ± 1.7
A204	Rhabdomyosarcoma	57.3 ± 3.5	57.0 ± 5.5
Nalm6	Pre-B cell	28.4 ± 1.2	24.2 ± 1.4
Jurkat	T cells ($IL2R^-$)	18.1 ± 1.3	17.6 ± 1.1
HUT102	Activated T cells ($IL2R^+$)	21.0 ± 2.0	20.8 ± 1.5
2B4	Murine T cell	89.7 ± 3.2	90.0 ± 13.7

The number of cells was determined after 72 hr of cells in culture. The results are expressed as the means of 3–4 experiments performed for each cell line ISE.

Figure 4. Induction of apoptosis in Colo 205 cells by GnRH-Bik/Bax/Bak chimeric proteins. FACS analysis (**A**) control cells (1); cells exposed to: GnRH-Bik (2); GnRH-Bak (3); or GnRH-Bax (4). The various chimeric proteins were added at a concentration of 5 ug/ml for 24 hr. Samples were FACS analyzed for DNA content (x) as a function of cell number (y), as described in Materials and Methods. M1 represent the percentage of cells in the sub-G1 phase; determined as the apoptotic cells. (**B**) Percentage of apoptotic cells ■ and of cells in S-phase △ as a function of increasing amounts of GnRH-Bik added for 24 hr.



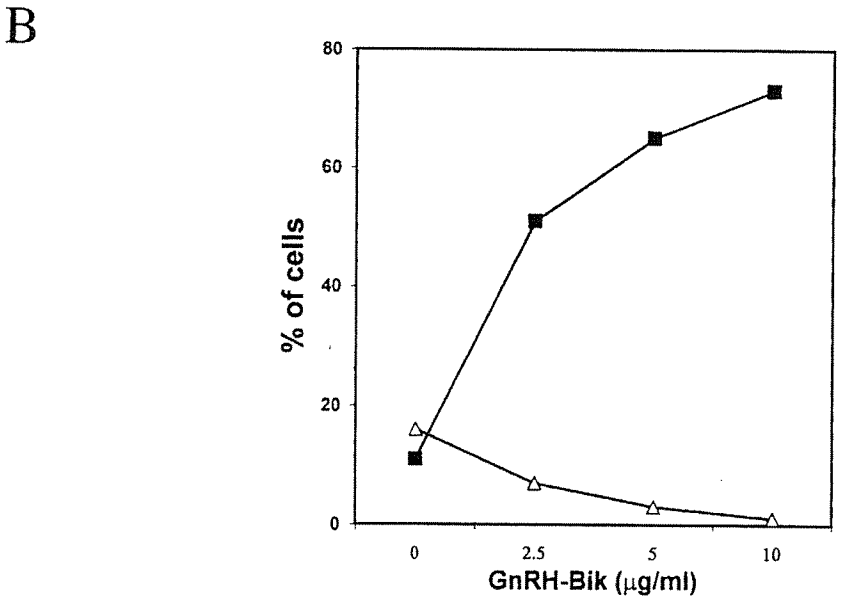


Figure 5. Morphology of adenocarcinoma target cells treated with GnRH-Bik. Cells were exposed to GnRH-Bik (5 μg/ml) or a control irrelevant chimera (Fc-Bak, 5 μg/ml) for 72 hr. Micrographs $\times$ 200.

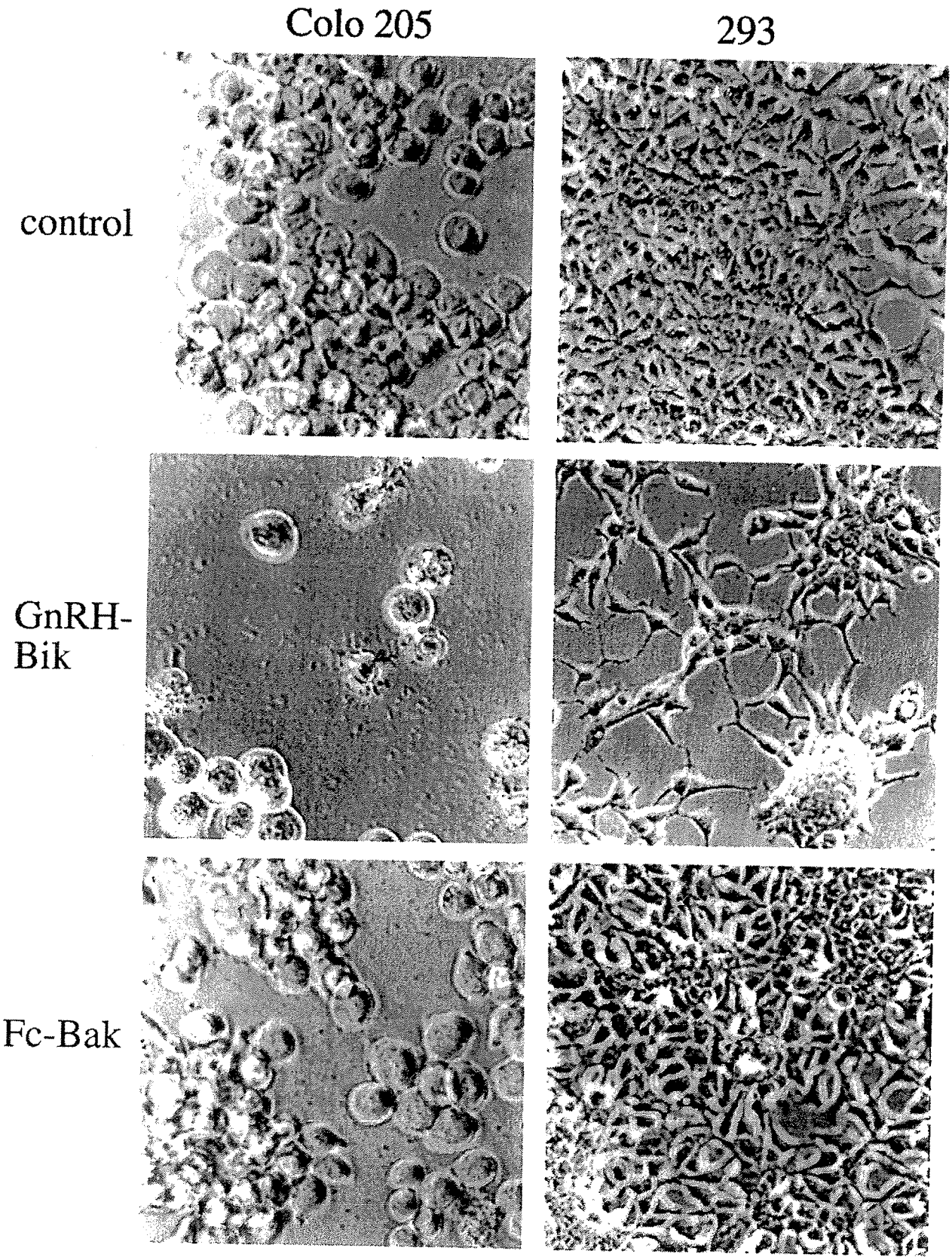
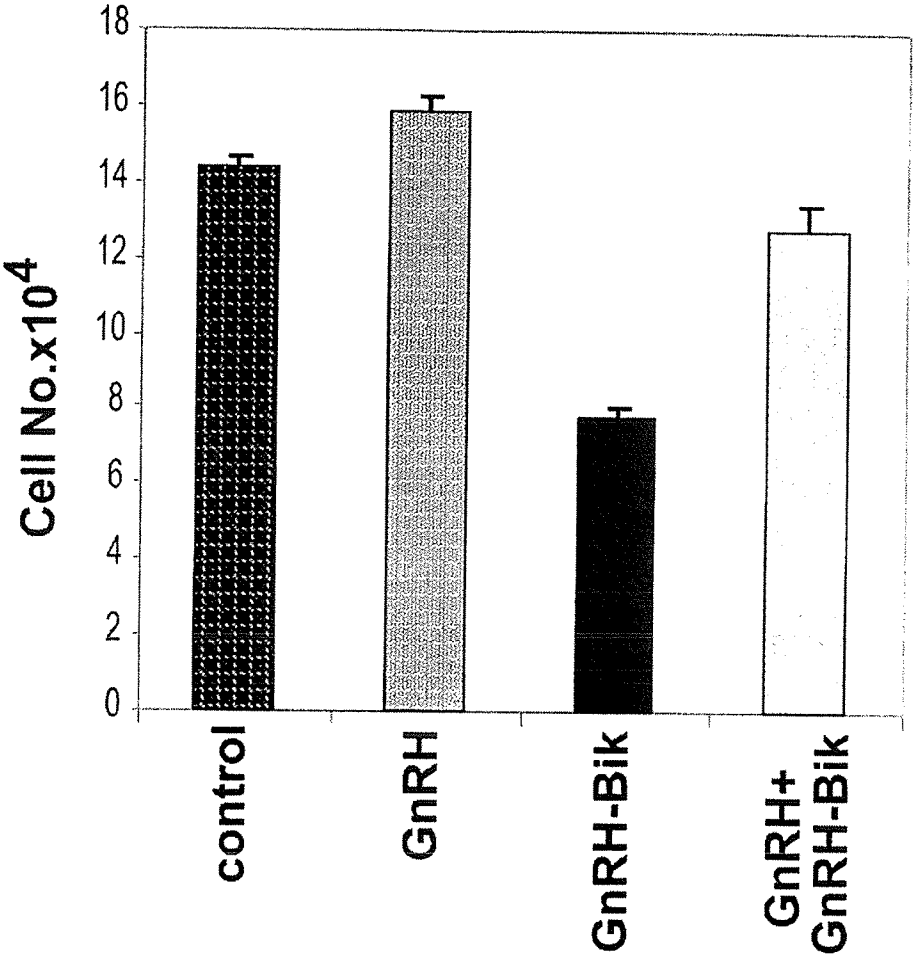


Figure 6. Competition of GnRH-Bik activity by GnRH. Colo205 cells were incubated with 35 μg (1.5×10^{-4}M) of GnRH in a standard cell growth assay (see Figures 2 and 3).



Expression of the Bik protein in various adenocarcinoma cell lines

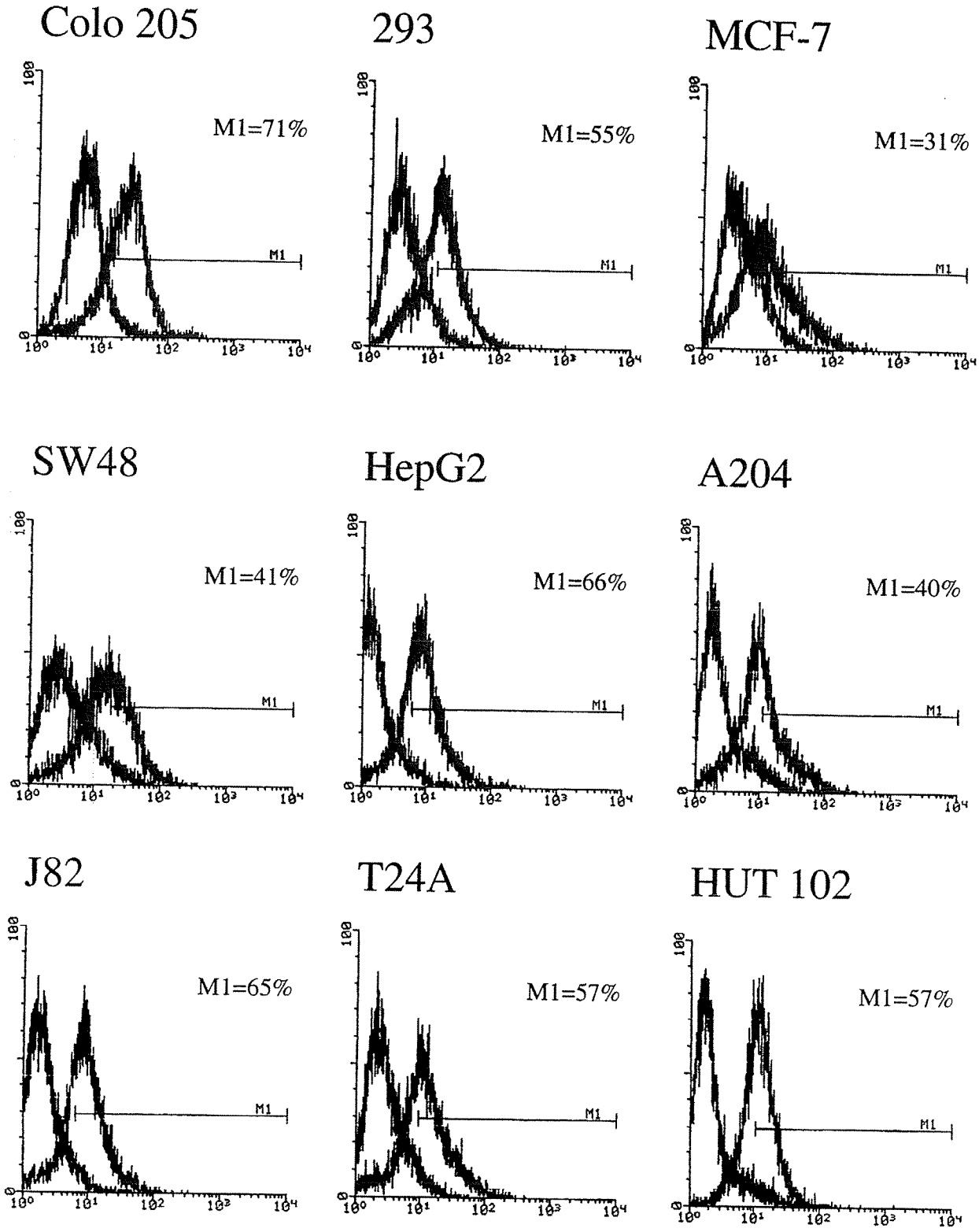
It was recently reported that the "dead gene" Bik is expressed, at various levels, only in a restricted subset of human tissues, mainly in epithelial cells.[14] In addition, Bik-mRNA appears to be expressed exotopically in some tumor-derived cell lines.[14] To determine whether the variability in the response of the different adenocarcinoma cell lines to GnRH-Bik is related to the levels of endogenous Bik, we measured the levels of Bik protein by FACS analysis. All the tested cell lines expressed the Bik protein, but at variable levels (Figure 7). However, there was no significant difference between target and non-target cells in the level of the Bik protein.

Discussion

To date, the immunogenicity of targeting chimeric proteins (or immunotoxins), especially that of the toxin portion of the chimera, constitutes a problem to which no practical solution has been found. Approaches to deal with this problem are the use of immunosuppressant drugs, to change/humanize the toxin moiety of the chimeras or to make or to find non-immunogenic toxins. Very recently we reported the development of a new prototype of human chimeric proteins, taking advantage of human apoptosis-inducing proteins such as Bax, as novel killing components, instead of bacterial or plant toxins. The first human chimeric protein prototype, IL2-Bax, specifically targeted IL2-receptor expressing cells and induced cell-specific apoptosis.[5] In the present study we used both another targeting moiety, a GnRH analog, and a different member of the Bcl-2 family, the Bik protein, which represents the "BH3-only-domain" subclass of this family.

GnRH-Bik efficiently inhibited the cell growth of target adenocarcinoma cell lines (Figures 2,3,6), leading to cell death by inducing, most probably, apoptosis within the target cells (Figures 4, 5). The same specificity of response was observed when we constructed and tested additional Bcl-2 family members, the Bax and Bak proteins, fused to the GnRH targeting moiety (Figure 4 and

Figure 7. Levels of endogenous Bik protein measured by FACS analysis in various cell lines. Single color fluorescence flow cytometry analysis was performed using anti-Bik antibody as described in Materials and Methods. Left curve: control cells stained with FITC-conjugated second antibody alone; right curve: cells stained with anti-Bik and second antibodies (see Methods). M1 represents the percentage of each cell type stained with anti-Bik antibodies.



our unpublished results). All three Bcl2-proteins-based chimeric proteins, GnRH-Bik, GnRH-Bax and GnRH-Bak, target adenocarcinoma cells and induce apoptosis within the target cells.

In comparing the killing potency of the various Bcl-2 family proteins, the Bik protein proved to be a highly efficient death-effector protein, as compared with Bax and Bak (our unpublished results). These observations are in accordance with recent reports demonstrating that the so-called "BH-3-only proteins," such as Bik, are more potent death effectors than those proteins containing all BH-domains of the Bcl-2 family. However, as we used only partially purified preparations of the different chimeric proteins, variations in killing capacity may be attributed to differences in the expression levels of the chimeric proteins used. A more detailed study with highly purified GnRH-apoptosis inducing chimeric proteins is now underway.

Our results show that targeting the Bik protein via the GnRH binding site in the form of a chimeric protein is sufficient to induce apoptosis in the absence of any additional death stimulus.

While the mechanism of BH3-mediated cell death is still unclear, recent studies have demonstrated that the active state of BH3-only proteins may be regulated by a variety of specific regulatory mechanism, including post-transnational modification (such as phosphorylation of Bad, 15 and proteolytic processing of Bid, 16), regulated expression (such as of the DF5 protein, 17) and relocalization/sequestration of the proteins (such as for Bim protein, 18). As GnRH-Bik chimeric protein is efficiently causing cell death, our results may suggest that Bik protein introduced into the target cells, in the form of a chimeric protein, is already in its active form. Alternatively, Bik may be activated within the cell as if a death signal has been transmitted to the cell. The same may be true for the other two pro-apoptotic proteins-Bax and Bak. Introducing large amounts of the pro-apoptotic proteins within the cells may also activate endogenous proteins/mechanisms of apoptosis. Mechanisms by which those chimeric proteins are leading to cell death are still unknown and are under investigation.

Targeting a pro-apoptotic protein into cells in order to induce cell specific apoptosis raises the question: do the levels of the endogenous protein determine or contribute to the efficacy of the chimeric protein in inducing cell death? Measurements of the Bik protein levels (Figure 7) in the various cell lines revealed no correlation between the expression levels of the endogenous Bik and the efficacy of GnRH-Bik to induce apoptosis. This is most probably because by targeting the Bik protein in the form of a chimeric protein the cells are "floated" with the exogenous protein, regardless of the levels of endogenous protein. Thus, the potency of killing may be attributable to variations in the number of GnRH-binding sites on the different adenocarcinoma cell lines, and not to the level of endogenous Bik.

In our new chimeric proteins we fused the GnRH analog to three members of the Bcl-2 family differing in origin, size and mechanism of action from the PE toxin. Yet, we observed the same specificity toward adenocarcinoma cells as with the PE toxin.[1,2] Therefore, our results support our notion that adenocarcinoma cells express specific GnRH-binding sites, which are highly specific targets for our GnRH-based chimeric proteins. We have data suggesting that binding of the new chimeric proteins to these cells is not via the known human pituitary GnRH-receptor, but via an unknown GnRH or GnRH-like receptor/binding site, as we suggested previously.[1,2] Indeed, low affinity-high capacity GnRH binding sites distinct from the pituitary GnRH-receptor have been demonstrated in ovarian,[19] renal[20] and breast carcinoma.[21] Using radioligand binding studies, the presence of high-affinity binding sites for GnRH were also reported in the membranes of human ovarian, prostate, breast and endometrial cancer cell lines, as well as in the JAR choriocarcinoma cell line.[22] More recently, two new splice variants of the human GnRH receptor were reported and may be candidate(s) for our potential GnRH binding site.[23]

Although proteins such as Bik/Bax/Bak are intracellular proteins, they are of human origin and, as such, they are expected to display reduced immunogencity in human recipients. Furthermore, by killing target cells via the apoptotic pathway tissue damage or a systemic response is minimized. The apoptotic cells shrink and condense, while the organelles and plasma membranes retain their integrity. The dead cells are then rapidly phagocytized by neighboring cells or macrophages, disappearing before there is any leakage of their content.

Conclusion

Our present results show that the novel GnRH-Bik/Bax/Bak chimeric proteins have the ability to specifically target and efficiently kill human adenocarcinoma cells *in vitro*. Thus, apoptosis-inducing chimeric proteins such as GnRH-Bik appear to be promising candidates for the treatment of a variety of adenocarcinomas in humans. As in our system, cell-specific apoptosis is induced using both various targeting moieties (5, and present study) and different members of pro-apoptotic proteins of the Bcl-2 family (Bik, Bax, Bak), this novel approach could be commonly applied, using any molecule that binds a specific cell type fused to an apoptosis-inducing protein.

Acknowledgment

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Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions

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An amino acid sequence encodes a message that determines the shape and function of a protein. This message is highly degenerate in that many different sequences can code for proteins with essentially the same structure and activity. Comparison of different sequences with similar messages can reveal key features of the code and improve understanding of how a protein folds and how it performs its function.

THE GENOME IS MANIFEST LARGELY IN THE SET OF PROteins that it encodes. It is the ability of these proteins to fold into unique three-dimensional structures that allows them to function and carry out the instructions of the genome. Thus, comprehending the rules that relate amino acid sequence to structure is fundamental to an understanding of biological processes. Because an amino acid sequence contains all of the information necessary to determine the structure of a protein (*1*), it should be possible to predict structure from sequence, and subsequently to infer detailed aspects of function from the structure. However, both problems are extremely complex, and it seems unlikely that either will be solved in an exact manner in the near future. It may be possible to obtain approximate solutions by using experimental data to simplify the problem. In this article, we describe how an analysis of allowed amino acid substitutions in proteins can be used to reduce the complexity of sequences and reveal important aspects of structure and function.

Methods for Studying Tolerance to Sequence Variation

There are two main approaches to studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. This method has been extremely powerful for proteins such as the globins or cytochromes, for which sequences from many different species are known (*2–7*). The second approach uses genetic methods to introduce amino acid changes at specific positions in a cloned gene and uses selections or screens to identify functional sequences. This approach has been used to great advantage for proteins that can be expressed in bacteria or yeast, where the appropriate genetic manipulations are possible (*3, 8–11*). The end results of both methods are lists of active sequences that can be compared and analyzed to identify sequence features that are essential for folding or function. If a particular property of a side chain, such as charge or size, is important at a given position, only side chains that have the required property will be allowed. Conversely, if the chemical identity of the side chain is unimportant, then many different substitutions will be permitted.

Studies in which these methods were used have revealed that proteins are surprisingly tolerant of amino acid substitutions (*2–4, 11*). For example, in studying the effects of approximately 1500 single amino acid substitutions at 142 positions in *lac* repressor, Miller and co-workers found that about one-half of all substitutions were phenotypically silent (*11*). At some positions, many different, nonconservative substitutions were allowed. Such residue positions play little or no role in structure and function. At other positions, no substitutions or only conservative substitutions were allowed. These residues are the most important for *lac* repressor activity.

What roles do invariant and conserved side chains play in proteins? Residues that are directly involved in protein functions such as binding or catalysis will certainly be among the most conserved. For example, replacing the Asp in the catalytic triad of trypsin with Asn results in a 10^4-fold reduction in activity (*12*). A similar loss of activity occurs in λ repressor when a DNA binding residue is changed from Asn to Asp (*13*). To carry out their function, however, these catalytic residues and binding residues must be precisely oriented in three dimensions. Consequently, mutations in residues that are required for structure formation or stability can also have dramatic effects on activity (*10, 14–16*). Hence, many of the residues that are conserved in sets of related sequences play structural roles.

Substitutions at Surface and Buried Positions

In their initial comparisons of the globin sequences, Perutz and co-workers found that most buried residues require nonpolar side chains, whereas few features of surface side chains are generally conserved (*6*). Similar results have been seen for a number of protein families (*2, 4, 5, 7, 17, 18*). An example of the sequence tolerance at surface versus buried sites can be seen in Fig. 1, which shows the allowed substitutions in λ repressor at residue positions that are near the dimer interface but distant from the DNA binding surface of the protein (*9*). These substitutions were identified by a functional

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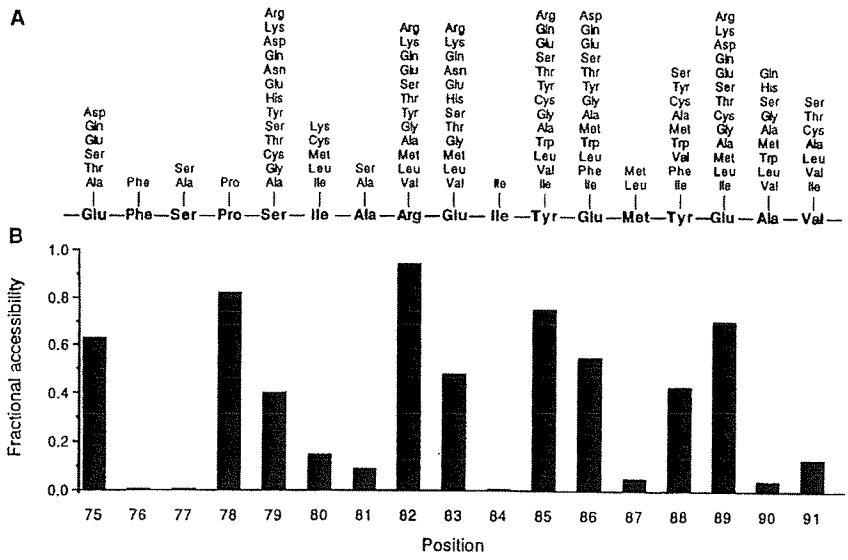

Fig. 1. (**A**) Amino acid substitutions allowed in a short region of λ repressor. The wild-type sequence is shown along the center line. The allowed substitutions shown above each position were identified by randomly mutating one to three codons at a time by using a cassette method and applying a functional selection (*9*). (**B**) The fractional solvent accessibility (*42*) of the wild-type side chain in the protein dimer (*43*) relative to the same atoms in an Ala-X-Ala model tripeptide.

selection after cassette mutagenesis. A histogram of side chain solvent accessibility in the crystal structure of the dimer is also shown in Fig. 1. At six positions, only the wild-type residue or relatively conservative substitutions are allowed. Five of these positions are buried in the protein. In contrast, most of the highly exposed positions tolerate a wide range of chemically different side chains, including hydrophilic and hydrophobic residues. Hence, it seems that most of the structural information in this region of the protein is carried by the residues that are solvent inaccessible.

Constraints on Core Sequences

Because core residue positions appear to be extremely important for protein folding or stability, we must understand the factors that dictate whether a given core sequence will be acceptable. In general, only hydrophobic or neutral residues are tolerated at buried sites in proteins, undoubtedly because of the large favorable contribution of the hydrophobic effect to protein stability (*19*). For example, Fig. 2 shows the results of genetic studies used to investigate the substitutions allowed at residue positions that form the hydrophobic core of the NH_2-terminal domain of λ repressor (*20*). The acceptable core sequences are composed almost exclusively of Ala, Cys, Thr, Val, Ile, Leu, Met, and Phe. The acceptability of many different residues at each core position presumably reflects the fact that the hydrophobic effect, unlike hydrogen bonding, does not depend on specific residue pairings. Although it is possible to imagine a hypothetical core structure that is stabilized exclusively by residues forming hydrogen bonds and salt bridges, such a core would probably be difficult to construct because hydrogen bonds require pairing of donors and acceptors in an exact geometry. Thus the repertoire of possible structures that use a polar core would probably be extremely limited (*21*). Polar and charged residues are occasionally found in the cores of proteins, but only at positions where their hydrogen bonding needs can be satisfied (*22*).

The cores of most proteins are quite closely packed (*23*), but some volume changes are acceptable. In λ repressor, the overall core volume of acceptable sequences can vary by about 10%. Changes at individual sites, however, can be considerably larger. For example, as shown in Fig. 2, both Phe and Ala are allowed at the same core position in the appropriate sequence contexts. Large volume changes at individual buried sites have also been observed in phylogenetic studies, where it has been noted that the size decreases and increases at interacting residues are not necessarily related in a simple complementary fashion (*5, 7, 17*). Rather, local volume changes are accommodated by conformational changes in nearby side chains and by a variety of backbone movements.

The Informational Importance of the Core

With occasional exceptions, the core must remain hydrophobic and maintain a reasonable packing density. However, since the core is composed of side chains that can assume only a limited number of conformations (*24*), efficient packing must be maintained without steric clashes. How important are hydrophobicity, volume, and steric complementarity in determining whether a given sequence can form an acceptable core? Each factor is essential in a physical sense, as a stable core is probably unable to tolerate unsatisfied hydrogen bonding groups, large holes, or steric overlaps (*25*). However, in an informational sense, these factors are not equivalent. For example, in experiments in which three core residues of λ repressor were mutated simultaneously, volume was a relatively unimportant informational constraint because three-quarters of all possible combinations of the 20 naturally occurring amino acids had volumes within the range tolerated in the core, and yet most of these sequences were unacceptable (*20*). In contrast, of the sequences that contained only

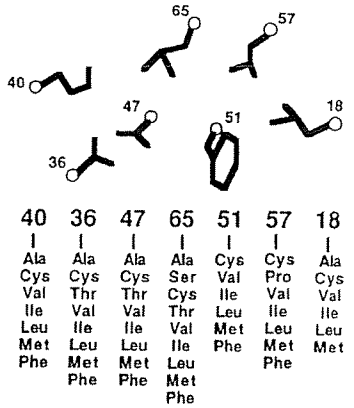

Fig. 2. Amino acid substitutions allowed in the core of λ repressor. The wild-type side chains are shown pictorially in the approximate orientation seen in the crystal structure (*43*). The lists of allowed substitutions at each position are shown below the wild-type side chains. These substitutions were identified by randomly mutating one to four residues at a time by using a cassette method and applying a functional selection (*20*). Not all substitutions are allowed in every sequence background.

the appropriate hydrophobic residues, a significant fraction were acceptable. Hence, the hydrophobicity of a sequence contains more information about its potential acceptability in the core than does the total side chain volume. Steric compatibility was intermediate between volume and hydrophobicity in informational importance.

The Informational Importance of Surface Sites

We have noted that many surface sites can tolerate a wide variety of side chains, including hydrophilic and hydrophobic residues. This result might be taken to indicate that surface positions contain little structural information. However, Bashford *et al.*, in an extensive analysis of globin sequences (*4*), found a strong bias against large hydrophobic residues at many surface positions. At one level, this may reflect constraints imposed by protein solubility, because large patches of hydrophobic surface residues would presumably lead to aggregation. At a more fundamental level, protein folding requires a partitioning between surface and buried positions. Consequently, to achieve a unique native state without significant competition from other conformations, it may be important that some sites have a decided preference for exterior rather than interior positions. As a result, many surface sites can accept hydrophobic residues individually, but the surface as a whole can probably tolerate only a moderate number of hydrophobic side chains.

Identification of Residue Roles from Sets of Sequences

Often, a protein of interest is a member of a family of related sequences. What can we infer from the pattern of allowed substitutions at positions in sets of aligned sequences generated by genetic or phylogenetic methods? Residue positions that can accept a number of different side chains, including charged and highly polar residues, are almost certain to be on the protein surface. Residue positions that remain hydrophobic, whether variable or not, are likely to be buried within the structure. In Fig. 3, those residue positions in λ repressor that can accept hydrophilic side chains are shown in orange and those that cannot accept hydrophilic side chains are shown in green. The obligate hydrophobic positions define the core of the structure, whereas positions that can accept hydrophilic side chains define the surface.

Functionally important residues should be conserved in sets of active sequences, but it is not possible to decide whether a side chain is functionally or structurally important just because it is invariant or conserved. To make this distinction requires an independent assay of protein folding. The ability of a mutant protein to maintain a stably folded structure can often be measured by biophysical techniques, by susceptibility to intracellular proteolysis (*26*), or by binding to antibodies specific for the native structure (*27, 28*). In the latter cases, it is possible to screen proteins in mutated clones for the ability to fold even if these proteins are inactive. Sets of sequences that allow formation of a stable structure can then be compared to the sets that allow both folding and function, with the active site or binding residues being those that are variable in the set of stable proteins but invariant in the set of functional proteins. The DNA-binding residues of Arc repressor were identified by this method (*8*). The receptor-binding residues of human growth hormone were also identified by comparing the stabilities and activities of a set of mutant sequences (*28*). However, in this case, the mutants were generated as hybrid sequences between growth hormone and related hormones with different binding specificities.

Implications for Structure Prediction

At present, the only reliable method for predicting a low-resolution tertiary structure of a new protein is by identifying sequence similarity to a protein whose structure is already known (*29, 30*). However, it is often difficult to align sequences as the level of sequence similarity decreases, and it is sometimes impossible to detect statistically significant sequence similarity between distantly related proteins. Because the number of known sequences is far greater than the number of known structures, it would be advantageous to increase the reach of the available structural information by improving methods for detecting distant sequence relations and for subsequently aligning these sequences based on structural principles. In a normal homology search, the sequence database is scanned with a single test sequence, and every residue must be weighted equally. However, some residues are more important than others and should be weighted accordingly. Moreover, certain regions of the protein are more likely to contain gaps than others. Both kinds of information can be obtained from sequence sets, and several techniques have

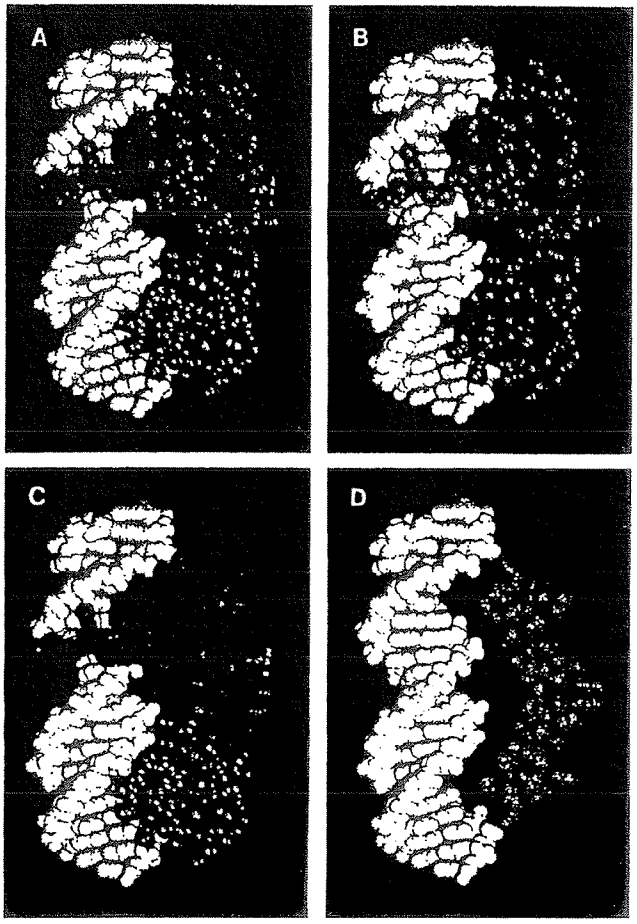

Fig. 3. Tolerance of positions in the NH_2-terminal domain of λ repressor to hydrophilic side chains. The complex (*43*) of the repressor dimer (blue) and operator DNA (white) is shown. In (**A**), positions that can tolerate hydrophilic side chains are shown in orange. The same side chains are shown in (**B**) without the remaining protein atoms. In (**C**), positions that require hydrophobic or neutral side chains are shown in green. These side chains are shown in (**D**) without the remaining protein atoms. About three-fourths of the 92 side chains in the NH_2-terminal domain are included in both (B) and (D). The remaining positions have not been tested. Data are from (*9, 14, 20, 27, 44*).

been used to combine such information into more appropriately weighted sequence searches and alignments (*31*). These methods were used to align the sequences of retroviral proteases with aspartic proteases, which in turn allowed construction of a three-dimensional model for the protease of human immunodeficiency virus type 1 (*29*). Comparison with the recently determined crystal structure of this protein revealed reasonable agreement in many areas of the predicted structure (*32*).

The structural information at most surface sites is highly degenerate. Except for functionally important residues, exterior positions seem to be important chiefly in maintaining a reasonably polar surface. The information contained in buried residues is also degenerate, the main requirement being that these residues remain hydrophobic. Thus, at its most basic level, the key structural message in an amino acid sequence may reside in its specific pattern of hydrophobic and hydrophilic residues. This is meant in an informational sense. Clearly, the precise structure and stability of a protein depends on a large number of detailed interactions. It is possible, however, that structural prediction at a more primitive level can be accomplished by concentrating on the most basic informational aspects of an amino acid sequence. For example, amphipathic patterns can be extracted from aligned sets of sequences and used, in some cases, to identify secondary structures.

If a region of secondary structure is packed against the hydrophobic core, a pattern of hydrophobic residues reflecting the periodicity of the secondary structure is expected (*33, 34*). These patterns can be obscured in individual sequences by hydrophobic residues on the protein surface. It is rare, however, for a surface position to remain hydrophobic over the course of evolution. Consequently, the amphipathic patterns expected for simple secondary structures can be much clearer in a set of related sequences (*6*). This principle is illustrated in Fig. 4, which shows helical hydrophobic moment plots for the Antennapedia homeodomain sequence (Fig. 4A) and for a composite sequence derived from a set of homologous homeodomain proteins (Fig. 4B) (*35*). The hydrophobic moment is a simple measure of the degree of amphipathic character of a sequence in a given secondary structure (*34*). The amphipathic character of the three α-helical regions in the Antennapedia protein (*36*) is clearly revealed only by the analysis of the combined set of homeodomain sequences. The secondary structure of Arc repressor, a small DNA-binding protein, was recently predicted by a similar method (*8*) and confirmed by nuclear magnetic resonance studies (*37*).

The specific pattern of hydrophobic and hydrophilic residues in an amino acid sequence must limit the number of different structures a given sequence can adopt and may indeed define its overall fold. If this is true, then the arrangement of hydrophobic and hydrophilic residues should be a characteristic feature of a particular fold. Sweet and Eisenberg have shown that the correlation of the pattern of hydrophobicity between two protein sequences is a good criterion for their structural relatedness (*38*). In addition, several studies indicate that patterns of obligatory hydrophobic positions identified from aligned sequences are distinctive features of sequences that adopt the same structure (*4, 29, 38, 39*). Thus, the order of hydrophobic and hydrophilic residues in a sequence may actually be sufficient information to determine the basic folding pattern of a protein sequence.

Although the pattern of sequence hydrophobicity may be a characteristic feature of a particular fold, it is not yet clear how such patterns could be used for prediction of structure de novo. It is important to understand how patterns in sequence space can be related to structures in conformation space. Lau and Dill have approached this problem by studying the properties of simple sequences composed only of H (hydrophobic) and P (polar) groups on two-dimensional lattices (*40*). An example of such a representation is shown in Fig. 5. Residues adjacent in the sequence must occupy adjacent squares on the lattice, and two residues cannot occupy the same space. Free energies of particular conformations are evaluated with a single term, an attraction of H groups. By considering chains of ten residues, an exhaustive conformational search for all 1024 possible sequences of H and P residues was possible. For longer sequences only a representative fraction of the allowed sequence or conformation space could be explored. The significant results were as follows: (i) not all sequences can fold into a "native" structure and only a few sequences form a unique native structure; (ii) the probability that a sequence will adopt a unique native structure increases with chain length; and (iii) the native states are compact, contain a hydrophobic core surrounded by polar residues, and contain significant secondary structure. Although the gap between these two-dimensional simulations and three-dimensional structures is large, the use of simple rules and sequence representations yields results similar to those expected for real proteins. Three-dimensional lattice methods are also beginning to be developed and evaluated (*41*).

Summary

There is more information in a set of related sequences than in a single sequence. A number of practical applications arise from an analysis of the tolerance of residue positions to change. First, such information permits the evaluation of a residue's importance to the function and stability of a protein. This ability to identify the essential elements of a protein sequence may improve our understanding of the determinants of protein folding and stability as well as protein function. Second, patterns of tolerance to amino acid substitutions of varying hydrophilicity can help to identify residues likely to be buried in a protein structure and those likely to occupy

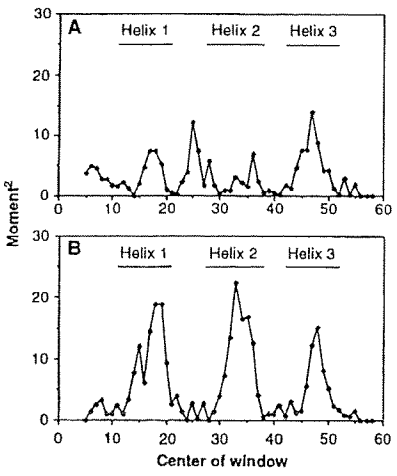

Fig. 4. Helical hydrophobic moments calculated by using (**A**) the Antennapedia homeodomain sequence or (**B**) a set of 39 aligned homeodomain sequences (*35*). The bars indicate the extent of the helical regions identified in nuclear magnetic resonance studies of the Antennapedia homeodomain (*36*). To determine hydrophobic moments, residues were assigned to one of three groups: H1 (high hydrophobicity = Trp, Ile, Phe, Leu, Met, Val, or Cys); H2 (medium hydrophobicity = Tyr, Pro, Ala, Thr, His, Gly, or Ser); and H3 (low hydrophobicity = Gln, Asn, Glu, Asp, Lys, or Arg). For the aligned homeodomain sequences, the residues at each position were sorted by their hydrophobicity by using the scale of Fauchere and Pliska (*45*). Arg and Lys were not counted unless no other residue was found at the position, because they contain long aliphatic side chains and can thereby substitute for nonpolar residues at some buried sites. To account for possible sequence errors and rare exceptions, the most hydrophilic residue allowed at each position was discarded unless it was observed twice. The second most hydrophilic residue was then chosen to represent the hydrophobicity of each position. An eight-residue window was used and the vectors projected radially every 100°. The vector magnitudes were assigned a value of 1, 0, or −1 for positions where the hydrophobicity group was H1, H2, or H3, respectively.

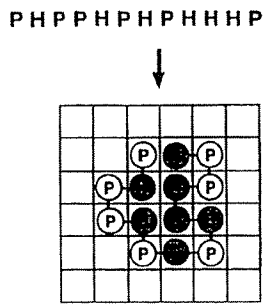

Fig. 5. A representation of one compact conformation for a particular sequence of H and P residues on a two-dimensional square lattice. [Adapted from (*40*), with permission of the American Chemical Society]

surface positions. The amphipathic patterns that emerge can be used to identify probable regions of secondary structure. Third, incorporating a knowledge of allowed substitutions can improve the ability to detect and align distantly related proteins because the essential residues can be given prominence in the alignment scoring.

As more sequences are determined, it becomes increasingly likely that a protein of interest is a member of a family of related sequences. If this is not the case, it is now possible to use genetic methods to generate lists of allowed amino acid substitutions. Consequently, at least in the short term, it may not be necessary to solve the folding problem for individual protein sequences. Instead, information from sequence sets could be used. Perhaps by simplifying sequence space through the identification of key residues, and by simplifying conformation space as in the lattice methods, it will be possible to develop algorithms to generate a limited number of trial structures. These trial structures could then, in turn, be evaluated by further experiments and more sophisticated energy calculations.

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APPENDIX 3

RELATED PROCEEDINGS APPENDIX

NONE